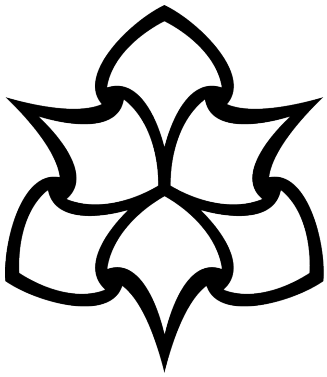

Orchid – Mycorrhiza relationships, propagation of terrestrial and epiphytic orchids from seed

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Abstract

Many orchids are rare and endangered due to habitat loss, degradation and human interference. This thesis aims to improve our understanding of orchid-mycorrhiza relationships and optimise methods for successful *in vitro* germination and development of terrestrial and epiphytic orchids. The research undertaken advances our understanding of orchids and has applications for conservation and horticulture.

Fungal specificity plays a key role in orchid distribution and the colonisation of habitats. This was investigated in the Australian terrestrial orchid, *Microtis media* R. Br. in Chapter 2. Experiments demonstrated low fungal specificity in germination *in vitro*, although later development was more specific, with one fungus inducing protocorm development through to adult plants. Fungal compatibility was tested in several Eurasian terrestrial orchids in Chapter 3. Varying degrees of specificity were observed, with *Anacamptis morio* (L.) R.M.Bateman, Pridgeon & M.W.Chase and *Dactylorhiza incarnata* (L.) Soó displaying low fungal specificity whereas *Serapias spp*. were more specific.

Orchids are commonly propagated without fungi using asymbiotic media. Asymbiotic and symbiotic methods were compared in two Eurasian terrestrial orchids in Chapter 4. In both cases, symbiotic methods resulted in higher germination and development. In *Anacamptis laxiflora* (Lam.) R. M. Bateman, Pridgeon & M. W. Chase, successful seedling establishment *ex vitro* was only achieved with symbiotic seedlings. Following this study, the effect of substrate rugosity and complexity on germination in asymbiotic and symbiotic culture of two Eurasian terrestrial orchids was investigated in Chapter 5. Substrate rugosity had a positive effect on germination and development in symbiotic cultures of *A. morio* and both asymbiotic and symbiotic cultures of *Dactylorhiza purpurella* (T.Stephenson & T.A.Stephenson) Soó.

The subtribe Pleurothallidinae is one of the largest in the Orchidaceae and little is known about their propagation requirements. A number species from different genera within this group were tested with different asymbiotic media and fungi isolated from plants in an *ex situ* collection in Chapter 6. Germination was

highest with two fungal strains but development of plantlets only occurred on asymbiotic media.

Ex situ study and propagation of myco-heterotrophic orchids poses some difficulties. A novel method is presented, using microcosms for the initiation of tripartite symbioses with tree seedlings, fungi and orchids in Chapter 7. Associations between tree seedlings, fungi and orchid seed were observed. Germination was not achieved with seed of *Neottia nidus-avis* (L.) Rich. The method provides a basis for further development with applications in a range of ecological studies.

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Dedicated to Emma, my family, Geoff Robson and all conservationists

“Paid a rhoi’r ffidil yn y tô!”

“There’s only one Planet Earth,  *please look after it”*

Chapter 1.

General Introduction

Background

Renowned for their flamboyant, highly variable flower forms and unique pollination methods, orchids are often described as the pinnacle of flower evolution. Employing numerous deceptive, obscure and rewarding methods to ensure successful pollination by passing insects and higher animals (Nilsson, 1992). The Orchidaceae are monocotolydonous plants which have an exceptional diversity in growth form and adaptations to their natural environments. They can be found growing as epiphytes, lithophytes, terrestrials and even rheophytes. Orchids are typically characterised by monopodial or sympodial growth forms that are largely erect, creeping or pendant (Dressler, 1981). As many epiphytic and lithophytic orchids are regularly subjected to periods of dryness, they often have evolved fleshy succulent leaves and roots. In addition, many utilise thickened stems known as pseudobulbs for water and nutrient storage. Terrestrial orchids may also deploy these survival strategies, as well as utilising other unique storage methods such as tuberoids, corms, pseudobulbs, fleshy roots and rhizomes (Dressler, 1981; Koopowitz, 2001). The roots of epiphytes, lithophytes and some terrestrials can be photosynthetic and are covered by an outer layer of spongy cells known as the velamen. This layer aids with water and nutrient absorption and is particularly noticeable in bark and twig epiphytes (Dressler, 1981). Some epiphytic orchids have taken the adaptation of photosynthetic roots to the extreme, having vastly reduced leaves or, in some species, no leaves at all. This is contrasted by the terrestrial mycoheterotrophic orchids which maintain a wholly subterranean existence until flowering (Dressler, 1981; Koopowitz, 2001).

The orchid family has a very wide and almost ubiquitous distribution, being found as far north as Alaska, Canada, Greenland, Scandinavia and Russia to as far south as the Southern tip of South America and Maquarie island in the Pacific (Dressler, 1981; Koopowitz, 2001). Their distribution, however, is non uniform

with a strong skew towards the tropics. Within the tropics, distribution varies widely with species abundance closely matching the high plant biodiversity hotspots delineated by Barthlott *et al.*, (1996) and Myers *et al*., (2000). The Andes region of Colombia and Ecuador is the richest places on earth for orchid diversity, with estimates of around a quarter of all known orchid species found there. Other areas of particular importance include: the mountains of the narrow neck of meso-America, Madagascar, Indo-China, Southwest China, Sumatra, Borneo, New Guinea, and temperate Southwest Australia (Cribb *et al*., 2003).

With varying estimates of between 17,500 – 35,000 species (Garay & Sweet, 1974; Gentry & Dodson, 1987; Mabberley, 1997; Cribb *et al*., 2003; Zotz, 2013; Christenhusz & Byng, 2016; WCSP, 2017), with the latter most estimating > 28,000 species (WCSP, 2017). Roughly two thirds of these are epiphytes and lithophytes and the remaining third terrestrial (Swarts & Dixon, 2009a). Orchidaceae can be considered one of the largest plant families accounting for c. 8% of angiosperm species diversity (Chase *et al*., 2003, 2015; Willis, 2017; Fay, 2018). Orchids are thought to be an ancient plant group. Their current distribution suggests an origin that likely predates the break-up of Gondwanaland, 125 million years ago and they are still actively evolving (Raven & Axelrod, 1974; Chase, 2001; Cribb *et al*., 2003).

One way in which orchids have evolved differently to other plants is their relationship with mycorrhizal fungi. Their specialisation has resulted in a heavy reliance on mycorrhizal fungi for at least some stages in their life cycle, most notably during germination. This is largely due to the minute size of orchid seeds which limit the amount of nutrient storage needed for successful germination and subsequent protocorm development. Although orchid seeds are capable of germinating in water, they are highly reliant on being infected by an appropriate fungus for further development to occur (Rasmussen, 2006). Infection by a suitable fungus induces a myco-heterotrophic response from the orchid embryo, where the germinating embryo digests invading fungal tissue, obtaining carbon and nutrients, enabling protocorm formation and potentially further plant development. Once germinants have produced their first chlorophyllous tissues they can synthesise their own energy by photosynthesis and their dependence on mycorrhizal fungi decreases (Rasmussen, 2006).

Mycorrhizae are a critical component in all ecosystems and play a key part in nutrient cycling. Mycorrhizae are the symbiotic association between plant roots and fungi and serve as a connection point between plants and the subterranean mycological world. The symbiosis is typically mutualistic, with a few weakly pathogenic associations (Kirk *et al*., 2001). Ninety-five percent of all plant species examined to date have been found to have active mycorrhizal relationships (Trappe, 1987), demonstrating the broad scale of mycorrhizal activity that is present in nature. The mycorrhizal association is beneficial to both partners as it increases the overall root surface area, resulting in greater water and nutrient absorption. This can allow for colonisation of areas which may not have been hospitable had the plants and fungi not developed this association (Ennos & Sheffield, 2000).

The mutualistic association between plants and fungi likely occurred because each experienced nutrient deficiencies that in turn led to the exchange of nutrients (Smith & Read, 2000). As autotrophs, plants photosynthesise above the soil's surface capturing the sun's light and converting it into usable forms such as carbon (C) containing sugars. In contrast, soil fungi are particularly efficient at mobilising key nutrients such as phosphorus (P), nitrogen (N) and trace elements. This means both organisms can effectively act as sinks for their sequestered elements and may become deficient in other nutrients. The developed symbiotic relationship allows complementary nutrient acquisition and exchange. These associations are initiated by sugar rich plant root exudates which attract fungal mycelia to colonise the roots of the plant, in some cases linking them together in a mycorrhizal network with other plants allowing for interplant nutrient exchange (Selosse *et al*., 2006). Rasmussen (2006) stated that "The orchid family is the only large group of higher plants that makes consistent use of an alternative nutritional system''. The degree of fungal reliance can vary considerably between different orchid genera and even within species due to diversity of habitat factors (Roberts & Kingsley, 2008). Unlike other major types of mycorrhizae, orchid mycorrhizas provide the plants with energy so still play a vital part in adult orchid survival (Rasmussen, 2006). Orchids with reduced rhizomes and roots are more reliant on the provision of nutrients by fungi and may even rely to a greater extent on myco-heterotrophy.

Orchid reproduction, germination and establishment requires highly specific interactions with pollinators and mycorrhizal fungi. These specialisms can often result in species with narrow geographic ranges which are limited to certain habitats (Cribb *et al*., 2003). As a result, orchid populations are easily impacted by anthropogenic activities such as habitat loss, overexploitation and climate change (Swarts & Dixon, 2009a). Many orchids are threatened by large-scale over-harvesting for medicinal (e.g. traditional Chinese medicine (Liu *et al*., 2014)), edible (e.g. Salep (Sazak & Ozdener, 2006; Ghorbani *et al*., 2014; Kemeç *et al*., 2015)) and horticultural (Koopowitz, 2001; Rannback, 2007; Hinsley *et al*., 2015) trades. The IUCN Global Red List currently includes assessments for 948 orchid species, of these 56.5% are threatened, the highest number of threatened species for any plant family (IUCN 2017). However, this leaves c. 27,000 species to be assessed for the Global Red List (Fay, 2018). Of the species recorded as extinct by the World Conservation Union (IUCN.1999) roughly half are terrestrial (Swarts & Dixon, 2009a). With the greater proportional risk faced by this group it is vital that effective conservation strategies are implemented to prevent further loss. In order to preserve orchid species and their diversity, active *in situ* conservation and, where necessary, *ex situ* conservation efforts are required. The study and optimisation of *ex situ* propagation methods is essential for preserving many rare species, especially where populations have become unviable due to severe habitat reduction, fragmentation or collection pressures (Swarts & Dixon, 2009b).

Key issues – fungal specificity and compatibility

Research into *ex situ* propagation must involve study of the orchid-mycorrhiza relationship, as it plays a significant part in orchid reproduction; from germination to the establishment of adult plants (Rasmussen, 2006). Fungal specificity in orchids is a significant area for consideration due to the variances in orchid – fungi compatibility. Studies on chlorophyllous Australian (Warcup, 1981; 1988; Bonnardeaux *et al.,* 2007) and Eurasian terrestrial orchids (Kulikov & Filippov, 2001) have shown that certain orchids, many of which are often characteristic of wasteland and disturbed areas, form associations with a wide range of mycorrhizal fungi. Other orchid genera proved to be highly specific in their fungal partners often only germinating with one particular fungal isolate. Bonnardeaux *et al.,* (2007) and Bidartondo & Read (2008) have shown that orchid fungal

specificity can change throughout an orchid's lifecycle. Where a narrow mycorrhizal range is observed during seedling development compared to the far more promiscuous associations during germination and mature stages.

In Chapter 2 of this thesis I wanted to examine the current notions of orchid-fungal specificity and compatibility by studying the common Australian terrestrial orchid *Microtis media* R. Br., and determine whether observations made by other researchers *in situ* also apply *in vitro.* Fungal specificity and compatibility in the orchid was examined by studying its germination and development *in vitro* on symbiotic H1 oat media (Clements *et al*., 1986, modified by Rasmussen *et al*., 1990a) with five orchid and non-orchid mycorrhizal fungi. The tested fungi had either similar or differing geographic distributions to *M. media* and comprised: *Ganoderma australe* (Fries) Patouillard complex, *Phialocephala fortinii* C.J.K. Wang & H.E. Wilcox, *Rhizoctonia repens* N. Bernard, *Rhizoctonia solani* Kühn and *Sebacina vermifera* Oberwinkler. It should be noted that the results of *in vitro* germination experiments should be interpreted with due caution, as the conditions *in vitro* and resultant fungal growth may not be directly comparable to those found *in situ*.

In Chapter 3 of the thesis, an experiment was devised to test the effectiveness of symbiotic propagation and examine fungal compatibility in four Eurasian tuberous terrestrial orchids from the subtribe Orchidinae: *Anacamptis morio* (L.) R.M.Bateman, Pridgeon & M.W.Chase, *Dactylorhiza incarnata* (L.) Soó, *Serapias bergonii* E.G.Camus, *Serapias parviflora* Parl. They were sown on H1 symbiotic media with five fungi. The fungi included two species of the well-known orchid symbiont genus *Rhizoctonia*: *Rhizoctonia repens* N.Bernard, *Rhizoctonia solani* Kühn and three strains of mycorrhizal fungi isolated from terrestrial orchids: A36, B1 (*Ceratobasidium sp.* Anastamosis Group C), Q414, and an asymbiotic control using Malmgren (1996) modified media (Malmgren & Nyström, 2014).

It should be noted that to determine true symbiosis between orchids and fungi, plant tissues, such as roots or protocorms, must be sectioned and the presence of fungi inside cortical cells confirmed both observationally and through subculture of fungal peletons excised from cortical cells. These procedures were, however, not performed in these chapters due to the need for suitable numbers

of replicates to undertake successful statistical analysis. Additionally there was a need to produce as many adult plants as possible, and in many treatment there were insufficient numbers of protocorms or plantlets to warrant sacrificing them for subsequent sectioning. In this work, symbiotic germination is defined as the germination of orchid seed in combination with a fungus on culture plates *in vitro*. Where orchid seed and protocorms are observed to interact with fungal hyphae, forming protocorms with rhizoids. These results are, where appropriate, corroborated with sowings on non-fungal control treatments.

Key issues – asymbiotic vs symbiotic propagation

Terrestrial orchids are commonly propagated from seed using agar based nutrient media, which can be symbiotic (inoculated with a symbiotic fungus) or asymbiotic (without fungi). Symbiotic propagation can be achieved using a variety of media, but the oat based media developed by Clements & Ellyard (1979) is most commonly used in scientific investigations (Clements *et al.,* 1986; Muir, 1989; Rasmussen, 1990a, 1990b; Zettler & Hofer, 1998; Batty *et al*., 2001; Zettler *et al.,* 2001; Wood & Ramsay, 2004; Batty *et al*., 2006a, 2006b; Bonnardeaux *et al.,* 2007) and by amateur hobbyists. Propagation via asymbiotic methods similarly uses agar based media, however, the nutrients which fungi would provide in symbiotic cultures are instead included in the media. In its simplest form, asymbiotic media is a mixture of sugars and fertiliser solution with the addition of gelling agent. The same basic nutrients which are needed for plant growth such as Nitrogen (N), Phosphorus (P), Potassium (K) and Magnesium (Mg) are provided along with varying amounts of trace elements, amino acids, vitamins, photochemicals and other complex organic substances (Seaton *et al.,* 2011). There are a great number of different formulations available (Rasmussen, 2006), though many orchid genera and species only germinate on a narrow range of asymbiotic media (Seaton *et al.* 2011).

There is much debate as to which is the best method of propagation. Since its development in the 1920s, asymbiotic media has seen widespread use and success, producing large numbers of plants without the additional effort of fungal isolation and the provision of suitable conditions for fungal symbiosis. This has left many to think symbiotic propagation methods are antiquated and of no effective use (Rasmussen, 2006). However, Seaton *et al.* (2011) state that when

combined with the appropriate fungi, some terrestrial orchids can be grown much faster with higher rates of survival *ex vitro* than comparable asymbiotic methods.

In Chapter 4, I test the effectiveness of asymbiotic and symbiotic *in vitro* propagation methods with two European terrestrial orchid species: *Anacamptis laxiflora* (Lam.) R. M. Bateman, Pridgeon & M. W. Chase and *Ophrys apifera* Huds. Both orchids were sown on asymbiotic Malmgren modified media and symbiotic H1 media, with the B1 fungal isolate (*Ceratobasidium sp*. Anastamosis Group C) used for *A. laxifolia* and the unidentified FOA8 fungal isolate for *O. apifera*.

Orchid seeds can prove to be difficult to germinate, with different investigators reporting varying results which cannot always be replicated (Hughes, unpublished data; Rasmussen, 2006; Ponert *et al*., 2011). As a result of the observations made in Chapter 4, I postulated that there may have been an effect of substrate rugosity and complexity on the germination and development in symbiotic cultures. In Chapter 5, a study was designed to investigate this effect on two Eurasian terrestrial orchids: *Anacamptis morio* and *Dactylorhiza purpurella* (T.Stephenson & T.A.Stephenson) Soó. These orchids were sown on agar based media with either flat or cut treatments and to determine whether substrate complexity had an effect; inorganic and soil based media were also tested. These media treatments were split into asymbiotic and symbiotic treatments using either asymbiotic Malmgren modified media or symbiotic H1 media with the B1 fungal isolate (*Ceratobasidium sp*. Anastamosis Group C).

Key issue – propagation of Pleurothallid orchids

The orchid subtribe Pleurothallidinae (Pleurothallid orchids) is likely the most species rich of all orchid subtribes with more than 5000 species represented of the currently accepted 28,484 species in the family Orchidaceae (Karremans, 2016, WCSP, 2017). Pleurothallid orchids are Neotropical in distribution (Pridgeon, 1982; Müller *et al.,* 2003) and display greatest species richness in the Andean cloud forests (Pridgeon, 1982) where there is a high degree of endemism (Dodson, 2005; Higgins & Williams, 2009; Pérez-Escobar *et al*., 2017). The majority of this subtribe are epiphytes, with a number of lithophytes, rheophytes and terrestrial species (Pridgeon *et al.,* 2001; Higgins & Williams, 2009). Many species have narrowly restricted populations, occupying niche habitats that often

contain only a small number of individuals (Tremblay & Ackerman, 2001; Jost, 2004; Crain & Tremblay, 2012, Pérez-Escobar *et al*., 2013; Pérez-Escobar *et al*., 2017). As a result, many of these orchids are considered rare, threatened or endangered (Llamozas *et al*., 2003; Calderón-Sáenz, 2006; Millner, 2013) and even minor changes to their habitat can have negative impacts on their populations (Cribb *et al.,* 2003; Swarts & Dixon, 2009b).

Very little literature exists on the propagation of Pleurothallid orchids, with studies on asymbiotic techniques focusing on the genera: *Masdevallia* (Pedroza-Manrique, 2006; Lopez-Roberts, 2007; Clavijo, 2010) and *Restrepia* (Millner *et al.,* 2008, Millner, 2013). Fewer studies have investigated symbiotic propagation, although Otero and Bayman (2009) compared asymbiotic and symbiotic methods in *Lepanthes rupestris* Stimpson. Building on techniques developed in previous chapters, a study was conducted in Chapter 6 to help determine optimal propagation methods for six species from four genera of the subtribe Pleurothallidinae: *Masdevallia melanoxantha* Linden & Rchb.f.; *Masdevallia menatoi* Luer & R.Vásquez*; Masdevallia patchicutzae* Luer & Hirtz*; Pleurothallopsis microptera* (Schltr.) Pridgeon & M.W.Chase*; Restrepia contorta* (Ruiz & Pav.) Luer; *Stelis sp*. Peru. Seeds were sown using both asymbiotic and symbiotic methods. Asymbiotic sowings were made with all six species on half strength and in the case of *M. melanoxantha*; *M. menatoi*; *P. microptera* full strength asymbiotic Knudson C (Knudson, 1946), Sigma P6668 – Phytamax™ orchid maintenance medium and Vacin and Went (VW) (Vacin & Went, 1949) media. For *M. melanoxantha,* additional treatments containing 100ml/l of coconut water were included with half and full strength asymbiotic media. Symbiotic sowings were made on H1 media with fungi isolated from orchid roots of this subtribe growing *ex situ* in the National Plant Collection® of Pleurothallid orchids maintained by the North of England Zoological Society.

Key issue – propagation of myco-heterotrophic orchids

Myco-heterotrophic (MH) orchids are mostly achlorophyllous, with a few exceptions such as *Limodorum abortivum* (Linne), which still retains some chlorophyll in its stalk (Blumenfeld, 1935; Rasmussen, 2006). These orchids often have reduced rhizomes and roots as fungi solely provide for their nutrition (Rasmussen, 2006). Such orchids have been implicated in having a tripartite

relationship, involving a MH orchid, an ectomycorhizal fungi and a tree, representing the most advanced stage of orchid mycotrophy. It is now thought that these orchids are epi-parasitic consuming energy and nutrients from both tree and fungus (Leake, 2005; Roy *et al*., 2009). The majority of MH orchids are thought to be highly specific in their choice of fungal partners (Leake, 2004), although some have been shown to associate with a number of fungi, at least during germination (Umata, 1995).

Myco-heterotrophic orchids have been propagated successfully *ex situ* with fungi using *in vitro* techniques on agar based media (Umata, 1995; Umata, 1998a; Umata, 1998b), inorganic soils with a complex carbohydrate source (Yagame *et al*., 2007) and *ex situ* containers containing soil, utilising tripartite associations between orchid, fungi and ectomycorrhizal shrubs (Warcup, 1985). A highly fungal dependent mixotrophic (HFDM) orchid has also been propagated from seed. This was achieved by first utilising asymbiotic techniques to produce seedlings (Yamato & Iwase, 2008), followed by the formation of tripartite associations *ex situ*, utilising containers with inorganic soil, fungi and tree seedlings (Yagame & Yamato, 2012). The *in situ* seed packet method has been used successfully to germinate seeds and produce seedlings of MH (McKendrick *et al.,* 2000a, 2002) and HFDM orchids (Bidartondo and Read, 2008). In the case of McKendrick *et al.* (2000a) the resultant seedlings were removed and trialled in *ex situ* microcosm containers containing soil and tree seedlings from the habitat.

Although a wide range of methods have been used to propagate MH orchids, none have been used to germinate a wide range of MH and HFDM orchids to the extent that they can be successfully reintroduced. In Chapter 7, I demonstrate a method using *ex situ* microcosm containers for the potential initiation and investigation of tripartite symbioses between tree seedlings, fungi and orchids. Associations were attempted between tree seedlings of: *Fraxinus excelsior* L.*; Quercus robur* L.*; Tilia cordata* Mill., seed of the MH orchid *Neottia nidus-avis* (L.) Rich. and soil samples containing fungi from the orchid habitat.

Summary

Collectively, these studies provide us with an increased understanding of orchid-mycorrhizal associations. I have determined fungal specificity and compatibility *in vitro* in a number of orchid species and ascertained effective methods for the

germination and development of a range of orchids *ex situ*. This work will be useful for the horticultural production and conservation of orchids, both *in situ* and *ex situ* and will assist future scientific investigations, enabling a greater understanding of the ecology and physiology of these orchids.

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Chapter 2.

Symbiotic germination of the terrestrial orchid *Microtis media in vitro* is not dependent on fungal sympatry

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Abstract

Fungal compatibility *in vitro* was determined in the terrestrial Southwest Australian orchid *Microtis media* R. Br. by combining it with five mycorrhizal fungi from orchid and non-orchid sources: *Ganoderma australe* (Fries) Patouillard complex, *Phialocephala fortinii* C.J.K. Wang & H.E. Wilcox, *Rhizoctonia repens* N. Bernard*, Rhizoctonia solani* Kühn, *Sebacina vermifera* Oberwinkler on H1 oat media and a H1 non-fungal control. *Microtis media* displayed no specificity towards fungal partners during germination *in vitro*, geminating with all five fungal isolates. Further protocorm and plantlet development in *M. media* was highly specific, only occurring with a Belgian non-orchid strain of *R. solani.* Resulting in viable plants *ex vitro* that flowered after 33 months culture. New symbiotic relationships were recorded for the fungi *G. australe* and *P. fortinii* with *M. media.* This study shows that *M. media* demonstrates low fungal specificity during germination *in vitro* but displays high fungal specificity during later protocorm and plantlet development. With viable plants generated using a non-orchid isolated fungal symbiont.

Keywords Terrestrial orchid germination · Fungal compatibility orchids · Mycorrhizal Fungi · Symbiosis · Symbiotic propagation

Introduction

Orchids have complex and often highly specialised interactions with a multitude of organisms, including fungi. Such specialisation has resulted in a heavy reliance on mycorrhizal fungi for at least some stages in their life cycle, most notably during germination. However, as Rasmussen (2006) suggests, despite numerous studies investigating orchid–fungal associations and the propagation of orchids, the relationships between orchids and their mycorrhizal fungi still remain understudied in many species. Rannback (2007) notes the increasing horticultural interest in terrestrial orchids and suggests that the associated commercial demand makes them a key area for further research. Gaining a better understanding of orchid-fungal interactions in respect to orchid propagation could be beneficial for commercial selling purposes, making them cheaper to buy and more efficient to produce.

Some orchids have been found to associate with numerous mycorrhizal fungi (Hadley, 1970, Warcup, 1985, Muir, 1989, Bonnardeaux *et al*., 2007) including fungi from non-orchid sources (Hadley, 1970, Salman *et al*., 2002) whilst others, in particular myco-heterotrophic (MH) orchids, can be highly specific, often associating with just one species of fungus (Warcup, 1985; Lan *et al*., 1994; Umata, 1995). Therefore, a gradient of specificity appears to exist for orchid-mycorrhizal interactions. Many existing studies have focused on the fungal associations of one particular orchid species (Masuhara & Katsuya, 1994; Perkins *et al*., 1995; Hayama *et al*., 1999; McKendrick *et al.,* 2002; Illyés *et al.,* 2005; Yamato *et al.,* 2005; Yamato & Iwase, 2008; Chutima *et al.,* 2011a, 2011b). Following this work it is important to consider the associations of terrestrial orchid species on a broader scale. Examining a greater number of potential fungal-orchid associations and determining whether observations made by other researchers *in situ* also apply *in vitro.* This could be achieved by testing the viability of fungi from different geographic locations, isolated from orchid tissues at various life stages, orchid associated ectomycorrhizal plants, and other non-orchid sources. Such work could help to validate current theories on orchid-mycorrhizal specificity and compatibility, and may reveal important trends in relationships between orchids and fungal genera.

This study examines the *in vitro* fungal compatibility of the terrestrial chlorophyllous orchid *Microtis media* R. Br. with five reported mycorrhizal fungi: *Ganoderma australe* (Fries) Patouillard complex, *Phialocephala fortinii* C.J.K. Wang & H.E. Wilcox, *Rhizoctonia repens* N. Bernard*, Rhizoctonia solani* Kühn and *Sebacina vermifera* Oberwinkler using *in vitro* symbiotic methods (*sensu* Rasmussen *et al*., (1990a)).

Materials and Methods

Study species

Mature seeds of the Southwest Australian terrestrial orchid *Microtis media* were obtained from the Orchid Seedbank Project, Chandler, Arizona, USA in the summer of 2009. The fungal species used were: *Ganoderma australe, Phialocephala fortinii*, *Rhizoctonia repens, Rhizoctonia solani* and *Sebacina*

vermifera. The mycorrhizal fungi isolates originated from European microorganism banks. Isolates of *P. fortinii* CBS 443.86, *R. repens* CBS 298.32 and *S. vermifera* CBS 572.83 were acquired from the Centraalbureau voor Schimmelcultures (CBS) in the Netherlands, whilst *G. australe* MUCL 39657 and *R. solani* MUCL 47937 were obtained from the Belgian Co-ordinated Collections of Micro-organisms/Mycothèque de l'Université catholique de Louvain (BCCM/MUCL), Belgium. All of these fungi are known orchidaceous mycorrhizal fungi. *Ganoderma australe* was chosen as it has been shown to induce seed germination and plant formation in two Asiatic orchids, *Erythrorchis (Galeola) altissima* (Blume) Blume and *Erythrorchis ochobiensis* (Hayata) Garay (Umata, 1995; 1998a). The strain of *Ganoderma australe* used in this experiment originated from Malawi. *Phialocephala fortinii* was chosen as it has been found to associate with the North American terrestrial orchids *Calypso bulbosa* (L.) Oakes (Currah *et al*., 1987, 1997) and *Amerorchis rotundifolia* (Banks) Hulten (Currah *et al*., 1987) and induce germination and development in the Eurasian terrestrial orchid *Dactylorhiza praetermissa* (Druce) Soo (Zimmerman and Peterson, 2007). The particular strain used was isolated from the roots of *Pinus sylvestris* L. from Suonenjoki, Finland in 1975 (Wang & Wilcox, 1985).

Two species of the genus *Rhizoctonia* were used as they are one of the most commonly associated orchid mycorrhizas (Rasmussen, 2006). *Rhizoctonia repens* CBS 298.32 was isolated from the European terrestrial orchid *Anacamptis morio* (L.) R. M. Bateman, Pridgeon & M. W. Chase in the Netherlands and deposited by P. Vermeulen in 1934. This species has also been identified as a mycosymbiont of a number of Eurasian terrestrial orchids including *Dactylorhiza* (Kulikov & Filippov, 2001). The common plant pathogen *R. solani* and its corresponding teleomorph *Thanatephorus cucumeris* have also been found associated with *Dactylorhiza,* as well as broad range of other Eurasian and some Australian orchids (Downie, 1957, 1959; Rasmussen, 2006; Warcup 1981). The utilised strain of *R. solani* was isolated from *Brassica oleracea* L. in Belgium. *Sebacina vermifera* is a mycorrhizal associate of several chlorophyllous Australian terrestrial orchids (Warcup, 1971, 1981, 1988), including *M. media* (De Long *et al*., 2012). The utilised strain *Sebacina vermifera* CBS 572.83 was isolated from *Caladenia dilatata* R. Br.*,* isolate number 0750 by J. H. Warcup

(1981, 1988) and was found to germinate a number of Australian terrestrial species.

Experimental design

Fungal isolates were first plated onto H1 oat medium (Clements *et al*., 1986, modified by Rasmussen, 1990a) in 90 mm sterile petri dishes (Sterilin™, Thermo Fisher Scientific, Newport, UK) using aseptic technique in a laminar flow cabinet (Flowfast V 15P, Faster D:Group, Ferrara, Italy). Cultures were grown on in a dark, temperature controlled cabinet (LMS Series 4 Model 1200, LMS Ltd., Sevenoaks, Kent, UK) at 21±1 ºC, to obtain sufficient fungal material for subsequent inoculation of experimental plates.

Mature seeds were stored in greaseproof paper packets in an air tight container with silica gel at 3±1 ºC for 3 months prior to sowing. The orchid seeds were sown using the Royal Botanical Gardens, Kew orchid seed sowing protocol under aseptic conditions (M. M. Ramsay personal communication, RBG Kew). Approximately 30 – 50 seeds of each orchid species were placed in individual packets made from circular 90 mm diameter, no. 1 filter paper (Whatman International, Maidstone, Kent, UK). Each seed packet was sterilised in 0.5% w/v Sodium dichloroisocyanurate (SDICN) solution for 20 minutes. The seed packets then received three consecutive rinses in separate beakers of sterile water, with each rinse lasting 30 seconds. Following this, the seed was sown onto a 90 mm petri dish of H1 oat medium. Fungal inoculation was made by placing a small 1 mm^2 piece of fungus at the centre of each germination plate after the seeds were sown. The plates were then labelled, sealed using Parafilm M® (Bemis, Neenah, WI, USA) and placed in a lit, temperature controlled cabinet (LMS Series 4 Model 1200, LMS Ltd., Sevenoaks, Kent, UK) and incubated under 12/12 h light/dark photoperiod (20 µmol m^{-2} s^{-1}) at 21±1 ºC. Lighting was provided by two 36 W Philips Universal T8 cool white fluorescent lights (Philips Electronics UK, Guildford, Surrey, UK) vertically mounted behind frosted acrylic sheeting. Ten replicates of each orchid-fungal combination and a control on H1 oat medium without fungi were made, giving a total of 60 culture plates.

After 5 weeks, seed germination was measured by counting the number of protocorms on each plate under a stereo microscope. Protocorm formation was determined by the observation of a ruptured seed testa and the formation of

rhizoids (Rasmussen, 2006). Because the number of germinants per plate varied widely and was often zero, comparisons among fungal treatments were made using a customised resampling test written in R (R Development Core Team, 2015). This allowed the direct calculation of the probability that the differences in number of germinants were due to chance alone.

Upon development of leaves, germinants were individually transferred to double Magenta® GA-7 vessels (Magenta Corp., Chicago, USA) with 60 ml of H1 oat medium and cultured for a further 12 months until tubers were observed (Fig. 1a). Tubers were planted in 14 cm round plastic pots in a mixture of John Inness No. 1, horticultural sand and perlite to the ratio 1:1:1 and placed in a 10 cm deep plastic tray filled with horticultural sand. Trays were placed in a plant growth cabinet (Percival AR 66L, Percival Scientific, Perry, IA, USA) and given a Mediterranean growth cycle with 8 months at a diurnal temperature cycle of 16:12 ºC (±1 ºC) and a photoperiod of 10/14 h light/dark under Philips Universal T8 cool white fluorescent lights at 35 µmol m^{-2} s^{-1} followed by 4 months at 20:16 ºC (±1 ºC) and 14/10 h light/dark.

Results

Microtis media germinated on all five fungal isolates with four to nine plates of each combination containing protocorms. The highest number of protocorms on a single plate recorded was 25 with *S. vermifera* followed by 24 on *R. solani*, 14 on *R. repens,* 9 on *G. australe* and 5 on *P. fortinii*. Consistency of germination across replicate plates for each isolate was quite variable with germination on 4/10 with *S. vermifera,* 9/10 on *R. solani*, 5/10 on *R. repens,* 7/10 on *G. australe* and 5/10 on *P. fortinii.* No germination was observed on non-symbiotic H1 control plates.

Rhizoctonia solani gave the highest overall germination rate for *M. media* with a total yield of 67 protocorms. *Sebacina vermifera* gave a total number of 62 protocorms, *R. repens* a total of 36, *G. australe* a total of 26 protocorms, and *P. fortinii* yielded the lowest total number of protocorms at 17. Fungal growth was particularly strong in treatments with *G. australe* and *P. fortinii* with dense hyphal growth often growing over developing protocorms. The correlation between total

protocorms and number of plates showing germination is low at r = 0.20, however this is common for orchid germination experiments.

There were significant differences in the number of germinants per plate between *R. solani* and *G. australis* (P = 0.033), between *R. solani* and *P. fortinii* (P = 0.005), and between *S. vermifera* and *P. fortinii* (P = 0.047, Figure 1).

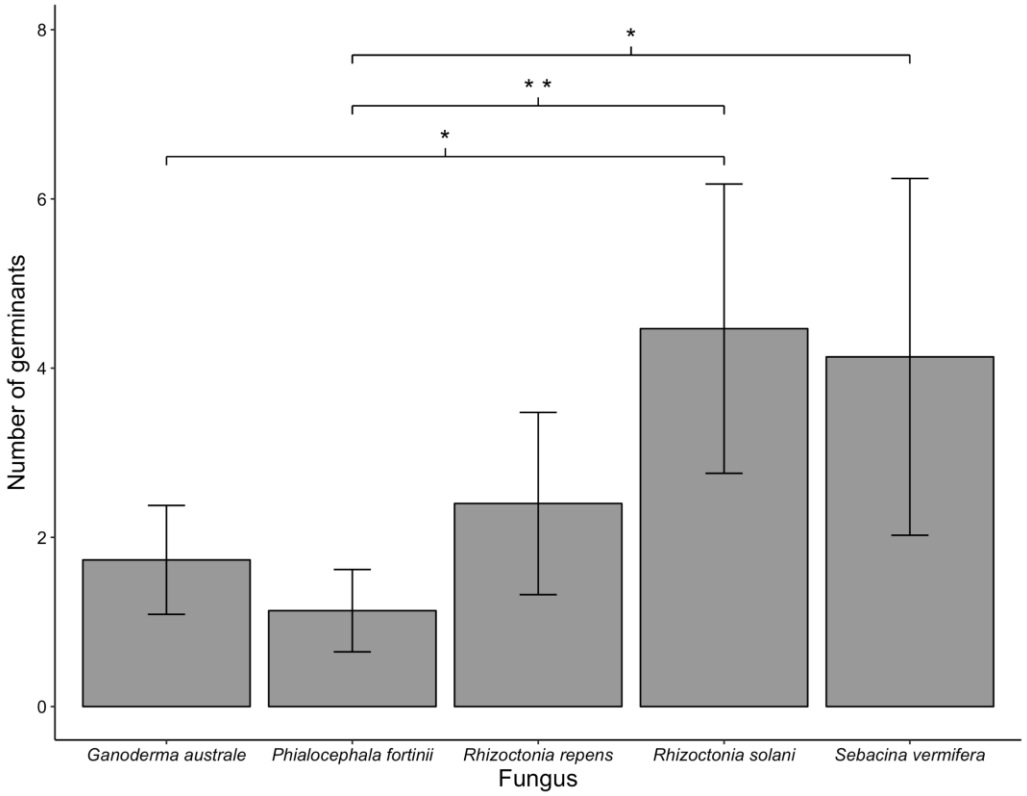

Figure 1. Number of protocorms (germinated seeds) of *Microtis media* on each of the fungal isolates at 5 weeks from sowing. Bars represent means +/- 1 SE. Lines indicate significant differences as determined by pairwise resampling tests. H1 media control was omitted due to lack of germination.

Protocorm development in *M. media* was observed microscopically and revealed the presence of several different development stages on each plate. The initial testa rupture and rhizoid formation (Figure 2a) was observed on all fungal treatments. Later shoot primordium (Figure 2b) and leaf formation (Figure 2c) were the most advanced developmental stages observed in *M. media* at 5 weeks, and only occurred in cultures with *R. solani*.

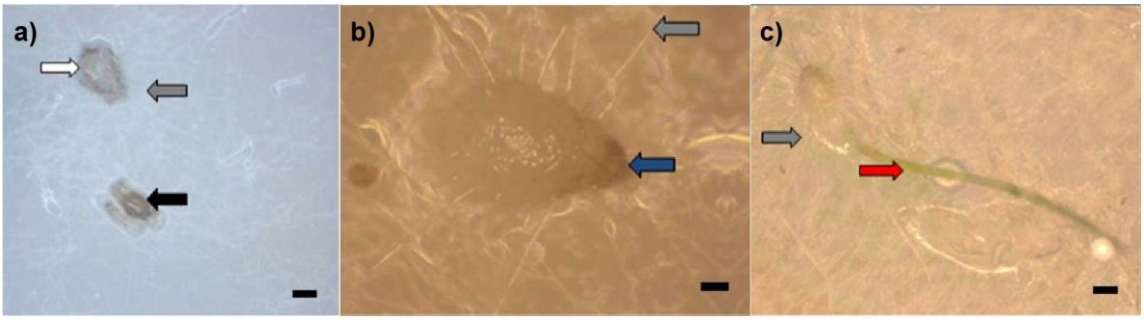

Figure 2. Germination and development at 5 weeks after sowing. **a)** Germinating seed of *Microtis media* colonised by *Ganoderma australe*, note ruptured testa (white arrow) and formation of rhizoids (grey arrow) interspersed by thinner hyphae (black arrows show seeds with swollen embryos which have not germinated). **b)** Protocorm of *Microtis media* colonised by *Rhizoctonia solani* which has formed rhizoids (grey arrow) and a shoot bud (blue arrow). **c)** Well developed potocorm of *Microtis media* colonised by *Rhizoctonia solani* with numerous rhizoids (grey arrow) and a green leaf (red arrow). *Bars*: 100 µm (**a**, **b**), 400 µm (**c**).

After 6 months of *in vitro* culture, all protocorms of *M. media* had died with exception of nine plantlets of *M. media* grown with *R. solani*. After 12 months further culture in Magenta® GA-7 vessels (Fig. 1a), three plants of *M. media* remained, all of which exhibited well-formed tubers and were removed from *in vitro* culture. Following 15 months culture in pots, flowers on two plants were observed (Figure 3).

Discussion

The results clearly demonstrate that different fungi caused differential germination in *M. media*. *Microtis media* germinated readily on all 5 fungal isolates and demonstrated low specificity towards fungal partners during germination. This concurs with Warcup's (1981, 1988) findings for other *Microtis* species, which germinated on a number of mycorrhizae isolated from Australian orchids. The isolate of *S. vermifera* used in this experiment originated from

Warcup's (1981, 1988) work, who referred to it as *S. vermifera* isolate No. 0750. It was found as an endophyte of the Australian terrestrial orchid *Caladenia dilatata* R. Br. and along with several other isolates of *S. vermifera* proved to be successful in germinating multiple *Caladenia* and *Microtis* species, *Eriochilus cucullatus* (Labill.) Rchb. f.*, Elythranthera sp*. and *Glossodia major* R. Br. The lack of further protocorm development with this fungal strain and *M. media* mirrors Warcup's (1981) results with *Microtis unifolia* (G.Forst.) Rchb.f.

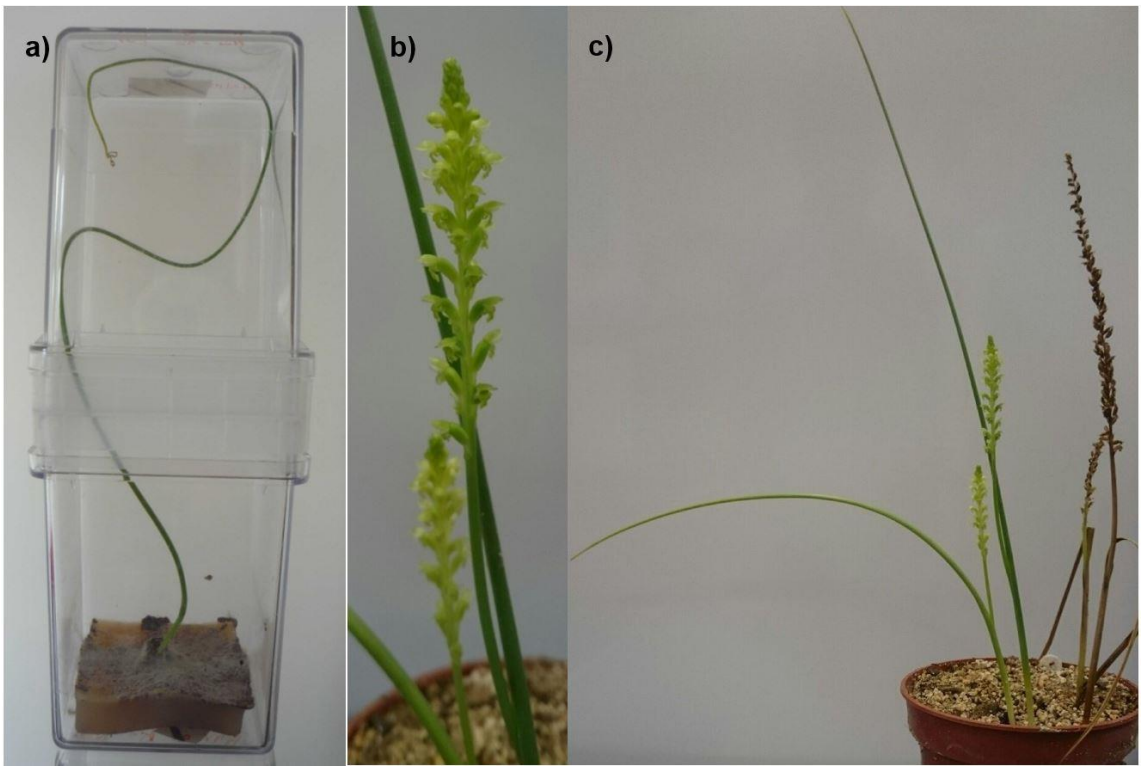

Figure 3. a) *Microtis media* plant with *Rhizoctonia solani* in double Magenta® GA-7 vessel prior to de-flasking, after 18 months culture. **b)** Close up of *Microtis media* flowers at 45 months culture (27 months culture *ex vitro*). **c)** Whole plant of *Microtis media* at 45 months culture (27 months culture *ex vitro*)

The data obtained for *M. media* also agrees with the findings by Bonnardeaux *et al.,* (2007) which looked at the diversity of mycorrhizal fungi among six terrestrial orchids, five of which were Australian and one an introduced South African species. They found *M. media* and the invasive South African orchid *Disa bracteata* Sw. to have the broadest webs of mycorrhizal fungi whereas *Diuris magnifica* D. L. Jones and *Thelymitra crinita* Lindl. had comparatively small webs of fungi. *Caladenia falcata* (Nicholls) M. A. Clem. &

Hopper and *Pterostylis sanguinea* D. L. Jones & M. A. Clem. were most specific in their fungal requirements germinating only with their own fungus. Not only did *M. media* and *D. bracteata* have relationships with diverse webs of fungi, but their fungal specificity was observed to increase over time. This was similarly supported by Warcup's (1981) findings with *M. unifolia*. These results indicate that a number of fungi can be involved at different stages in the orchid's lifecycle, where some may only induce protocorm formation and others can support further seedling growth. This is particularly relevant to the results of my experiment as protocorms of *M. media* were observed on all fungal isolates, yet shoot and leaf development was only observed with *R. solani.* This adds to the notion that protocorms of *M. media*, may exhibit increasing fungal specificity over time. These results should, however, be interpreted with due caution as the conditions *in vitro* and resultant fungal growth may not be directly comparable to those found *in situ*.

Further study by De Long *et al*., (2012) supported the notion that potentially invasive terrestrial orchids such as *M. media* may associate with a diversity of fungi which are common and widespread. Germination trials *in situ* and *ex situ* showed that several fungi enhanced seed germination in *M. media* but not other co-occurring orchid species, allowing *M. media* to tolerate a wider range of habitats. These traits potentially give *M. media* and other "weed-like (disturbance-tolerant rapidly spreading) orchids" (Bonnardeaux *et al.,* 2007) a competitive advantage over other co-occurring orchid species. They suggest that such "invasive orchids" may likely become more widely spread as they increasingly colonize novel habitats.

These results also add to the notion of fungal bottlenecks during orchid germination and development as observed by Bidartondo & Read (2008) in rhizomatous terrestrial species. Their investigation into fungal specificities of germinating seed, seedlings and mature plants of *Cephalanthera* and *Epipactis spp*. showed that seedlings of *Cephalanthera spp.* were more specific in their fungal partners, associating with a smaller range of fungi compared to germinating seed and adult plants. Whereas the fungi found to associate with *Epipactis atrorubens* (Hoffm.) Besser were similar in all three life stages, though seedling germination performance was affected by different fungal lineages. These works have brought about the notion that fungal bottlenecks can occur

during orchid germination and development; where a narrow mycorrhizal range is observed during seedling growth compared to the far more promiscuous germination and mature stages of the orchid's life cycle.

The results here demonstrate a number of previously unrecorded relationships between orchids and fungi. The fungus *G. australe* formed a symbiotic relationship with *M. media*, forming protocorms with numerous rhizoids and fungal hyphae. Umata (1995; 1998a) found *G. australe* also displayed a symbiotic capacity with the achlorophyllous, MH orchids; *Erythrorchis (Galeola) altissima* and *Erythrorchis ochobiensis*, inducing seed germination and plant formation. This proves that both chlorophyllous and achlorophyllous orchids are capable of associating with the same fungal species; however this remains to be shown in nature. The fungus *P. fortinii* demonstrated the capacity for symbiosis with the Australian terrestrial orchid *M. media,* inducing seed germination and protocorm formation. This fungus has only been observed previously as a mycosymbiont with North American holarctic orchid species, such as *Calypso bulbosa* (Currah *et al*., 1997), and is not native to Australia (ICAF, RBG Melbourne). It should be noted that fungal growth in *G. australe* and *P. fortinii in vitro* was not optimal for further protocorm development with high hyphal densities and strong growth, overgrowing developing protocorms.

The observation of germination *M. media* with *G. australe* and *R. solani* show that fungi from non-orchid sources can prove to be suitable symbionts at least under laboratory conditions. This concurs with observations made by Hadley (1970) with *Dactylorhiza purpurella* (T. Stephenson & T. A. Stephenson) Soó, *Dactylorhiza viridis* (L.) R. M. Bateman, Pridgeon & M. W. Chase, *Goodyera repens* (L.) R. Br, *Epidendrum ibaguense* Kunth and *Epidendrum radicans* Pav. ex Lindl. and Salman *et al*., (2002) with *D. fuchsii*. Studies by Masuhara & Katsuya (1994) with *Spiranthes sinensis* (Persoon) Ames *var. amoena* (M. Bieberstein) Hara and Perkins *et al*., (1995) with *Microtis parviflora* R. Br have shown that some terrestrial orchids display greater specificity in their fungal symbionts under natural conditions than when compared with *in vitro* studies trialling both orchid and non-orchid mycorrhizal fungi. The observations of Perkins *et al*., (1995) with *M. parviflora* are similar to those made with *M. media* in this study although the pathogenic strain of *R. solani* utilised with *M. media*

here did not kill germinating seeds but rather killed seedlings at a more developed stage once leaves were formed.

Germination was observed despite the fact that fungal isolates originated from different continents, *G. australe* originated from Malawi*, P. fortinii* from Suonenjoki in Finland, *R. repens* from the Netherlands, *R. solani* from Belgium and *S. vermifera* from Upper Sturt, South Australia. *M. media* germinated with all fungal isolates demonstrating that this species can form symbioses with geographically disjunct fungi, found on different continents and climatic zones to the associating orchid species. When considering the germination success of *M. media* with a broad web of mycorrhizal fungi over wide geographic distances, Bonnardeaux ***et al's*** (2007) description of *M. media* and *D. bracteata* as a "weed-like (disturbance-tolerant rapidly spreading) orchids'' seems particularly apt. Suggesting that *M. media* could be an invasive plant if introduced to other continents with Mediterranean climates as *D. bracteata* has already proven in Australia. It would thus be wise to curtail any cultivation or transport of these species in Mediterranean regions other than their native distributions. Additionally of interest was the ability of *R. repens* to induce germination in *M. media* despite the fact it has been in culture for over eighty years. This proves that long term axenic storage and culture does not necessarily negatively affect the symbiotic potential of orchid mycorrhizal fungi, though it would be of future interest to compare its effectiveness with a recently isolated strain.

Although flowering plants of *M. media* were obtained with this strain of *R. solani*, the considerable loss of seedlings, likely as a result of the pathogenicity of *R. solani,* do not support its use as a dependable symbiotic fungus. It was observed that seedling death was highest when leaves came into contact with the petri dish lid. This likely caused higher moisture levels on developing leaves as a result of condensation on the base of the petri dish lid. Potentially promoting abnormal fungal growth on leaves followed by later pathogenicity. Transfer of seedlings to deeper containers, prior to this occurring may solve this issue and make propagation using this method more commercially viable.

Further studies can help gain a better understanding of orchid-mycorrhizal interactions, increasing knowledge of vital ecosystem processes. With this knowledge it could be possible to achieve more success in germinating rare and

endangered orchid species, especially those that demonstrate a higher fungal specificity. The discovery of new biological functions for the fungi *G. australe* and *P. fortinii* as mycosymbiont's with *M. media* is significant. These findings suggest that greater study in this area could result in the discovery of many unknown interactions which take place under the soils surface, highlighting new and novel ways of propagating and reintroducing rare orchid species. Previous studies have mostly focused solely on terrestrial orchids from specific continental regions such as Eurasia, North America and Australia. Little has been done to look at fungal specificity and compatibility in orchids on a global scale. As a result there is still much to be learnt about the associations of orchids and their fungal partners and thus further justifies the need for research in this area.

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Chapter 3.

Assessing fungal compatibility in the germination and development of four terrestrial orchids of the subtribe Orchidinae

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Abstract

Fungal compatibility is an important factor in the symbiotic germination and development of orchids. This was investigated in four terrestrial orchid species from the subtribe Orchidinae: *Anacamptis morio* (L.) R.M.Bateman, Pridgeon & M.W.Chase; *Dactylorhiza incarnata* (L.) Soó; *Serapias bergonii* E.G.Camus; *Serapias parviflora* Parl. They were sown with five fungi, two species of the well-known orchid symbiont genus *Rhizoctonia*: *Rhizoctonia repens* N.Bernard; *Rhizoctonia solani* Kühn and three strains of mycorrhizal fungi isolated from terrestrial orchids: A36; B1 (*Ceratobasidium sp.* Anastamosis Group C); Q414; and an asymbiotic control using modified Malmgren media. Germination amongst species was varied; *D. incarnata* germinated on all treatments, with B1 proving most successful, *A. morio* germinated on all treatments with the exception of *R. solani*, developing to the furthest stage with A36 and B1 fungi, *S. bergonii* and *S. parviflora* displayed a higher fungal specificity, with *S. bergonii* only germinating with *R. repens* and Q414, *S. parviflora* was most specific only germinating with Q414, both species germinated most successfully on the asymbiotic control. The results of this study have long-term implications for orchid conservation and horticulture. Demonstrating effective propagation methods and fungal partners for producing seedlings of a number of temperate terrestrial orchids, and highlighting the differences in fungal specificity and compatibility between different orchid species within one subtribe.

Keywords Orchidaceae • Mycorrhizae • Fungal compatiibility • Germination • Conservation

Introduction

With the latest count at 28,484 species (WCSP, 2017) and a distribution on all continents, except Antarctica, the family Orchidaceae can be considered as one of the most diverse and widespread of all flowering plant families. The broad diversity associated with the Orchidaceae has resulted in many species developing specific relationships with insect pollinators and mycorrhizal fungi, some of which enable the colonisation of niche habitats. Such species may only have small or sporadic distributions and therefore, even minor changes to their

habitat could have a negative impact on orchid populations (Cribb *et al*., 2003; Swarts & Dixon, 2009b). As a result, a large number of terrestrial orchids are now considered rare, threatened or endangered (Whigham & Willems, 2003). The rarity of many terrestrial species, combined with a growing medicinal (Liu *et al*., 2014), edible (Sazak & Ozdener, 2006; Ghorbani *et al*., 2014; Kemeç *et al*., 2015) and horticultural (Koopowitz, 2001; Rannback, 2007) demand (Hinsley *et al*., 2015), highlights the need to develop effective propagation methods.

Terrestrial orchids are artificially propagated from seed using agar based nutrient media. These can be symbiotic, where a symbiotic fungus is cultured in association with orchid seed or asymbiotic, where seeds are sown without fungus on a sterile nutrient media. With many terrestrial orchids relying heavily on associations with mycorrhizal fungi, fungal compatibility trials are an important tool in finding effective fungal symbionts for propagating terrestrial orchids and gaining a better understanding of orchid-fungal specificity. Although orchid seeds are capable of germinating in water, they are highly reliant on being infected by an appropriate fungus for further development to occur (Rasmussen, 2006). Infection by a suitable fungus induces a myco-heterotrophic response from the orchid embryo, where the germinating embryo digests invading fungal tissue, obtaining carbon and nutrients, enabling protocorm formation and potentially further plant development (Rasmussen, 2006). These fungal associations can be specific, with orchids associating with only one or a group of closely related fungal species (Warcup, 1971, 1981, 1985; Kulikov & Filippov, 2001; Athipunyakom *et al.*, 2004; Leake, 2004; Bonnardeaux *et al.*, 2007; Yagame *et al.*, 2007), or unspecific, where orchids associate with many different fungal genera (Warcup, 1981, 1985; Kulikov & Filippov, 2001; Athipunyakom *et al.*, 2004; Bonnardeaux *et al.*, 2007; Bidartondo & Read, 2008; Yamato & Iwase, 2008, Hughes, Chapter 2).

The growing horticultural demand and rarity of many terrestrial orchids highlights the need to develop effective propagation methods. Asymbiotic media are widely and successfully used for commercial propagation, whereas symbiotic propagation is comparably neglected and is largely used by conservationists and amateur hobbyists (Zettler, 1997b; Kauth *et al*., 2008a; Seaton *et al.,* 2011). There is some debate as to which is the best method of propagation. For example, a study by Johnson *et al*. (2007) showed that asymbiotic propagation

can be more effective, producing large numbers of healthy plants without the trouble of isolating and culturing fungi. Other researchers (Muir, 1989; Anderson, 1991; Seaton *et al.,* 2011; Hughes, Chapter 4) suggest that when combined with appropriate fungi, some terrestrial orchid's exhibit higher growth rates and higher survival rates *ex vitro* when compared with asymbiotic methods.

Propagation using asymbiotic and symbiotic methods largely relies on agar based media. In their simplest form, these media are a mixture of sugars and fertiliser solution with the addition of a gelling agent. The basic nutrients that are needed for plant growth: Nitrogen (N), Phosphorus (P), Potassium (K) and Magnesium (Mg), are provided along with varying amounts of trace elements. In the case of asymbiotic media, these are often supplemented by amino acids, vitamins, phytochemicals and other complex organic substances (Seaton *et al.,* 2011). Symbiotic media, on the other hand, include insoluble carbohydrates which are made accessible to developing seedlings through the metabolism of the fungi (Rasmussen, 2006; Seaton *et al.* 2011). Orchidaceous mycorrhizal fungi can be isolated from living tissues such as protocorms, roots, rhizomes, tubers and corms. The process of extraction, isolation and testing of mycorrhizal fungi is a complicated process. The time and efforts required to obtain pure cultures of suitable fungal symbionts adds additional costs and constraints to the symbiotic method (Zettler, 1997a; Rasmussen, 2006). However, developing suitable media compositions for asymbiotic methods can prove to be equally difficult and time consuming. Different genera and species often demand varying amounts of mineral nutrients, sugars and other constituents for successful germination and seedling growth (Malmgren, 1988, 1989; Rasmussen, 2006).

The aim of this study was to ascertain the fungal compatibility and most effective propagation method for four orchid species from the subtribe Orchidinae by testing the suitability of five fungi: *Rhizoctonia repens* N.Bernard; *Rhizoctonia solani* Kühn; A36; B1 (*Ceratobasidium sp.* Anastamosis Group C) and Q414, along with an asymbiotic control using modified Malmgren (1996) media (Malmgren & Nyström, 2014). It was hypothesised that there would be different responses for each orchid species to each fungus tested with some fungi inducing no germination, others inducing germination but not further development and others inducing higher germination and more advanced development compared

to other treatments. Asymbiotic treatments in comparison would give positive germination results in all orchid species tested.

Anacamptis morio is listed as near threatened on the Red List of Vascular Plants for Great Britain (Cheffings *et al*., 2005) and Europe (Bilz *et al*., 2011) and may benefit from *ex situ* propagation followed by reintroduction efforts. Many species within these genera including *A. morio* are subject to heavy collection pressures from the Salep trade (Sazak & Ozdener, 2006; Ghorbani *et al*., 2014; Kemeç *et al*., 2015). Determining fungal compatibility in orchids can help validate current theories on orchid-fungal specificity and reveal important relationships between orchids and fungal genera. This study should help identify viable fungal symbionts for orchid propagation, potentially aiding production of these plants for horticulture and conservation of rare species within these genera.

Materials and Methods

Organismal details

For this study four orchid species from the subtribe Orchidinae were chosen based on availability of seed and Eurasian temperate to Mediterranean distributions. The first, *Anacamptis morio* (L.) R.M.Bateman, Pridgeon & M.W.Chase is a tuberous geophyte, producing a wintergreen rosette and aestivating as globose rootless tubers. It is Euro-Mediterranean in distribution, growing as far north as Britain and southern Norway east to Iran. The second, *Dactylorhiza incarnata* (L.) Soó is a tuberous geophyte, producing summer-green leafy shoots, which originate from palmately divided tubers. It is Euro-Siberian in distribution and found in Spain, Britain, Ireland and Scandinavia in the west across to China and the Russian Far East. The third, *Serapias bergonii* E.G.Camus is similar in growth morphology to *A. morio* and is found in the eastern Mediterranean. The fourth, *Serapias parviflora* Parl. is identical in growth morphology to *S. bergonii* but is Mediterranean-Atlantic in distribution, from the Canary Islands in the southwest, north to Brittany, France and east to Cyprus (Delforge, 2006; Rasmussen, 2006).

Two species of *Rhizoctonia* were used in this study, as they are one of the most commonly associated orchid mycorrhizas (Rasmussen, 2006). These were: *Rhizoctonia repens* and *Rhizoctonia solani*. *Rhizoctonia repens* CBS 298.32 was obtained from the Centraal Bureau voor Schimmelcultures, Netherlands and was isolated in 1934 by P. Vermeulen from *Anacamptis morio* in the Netherlands. It has been found to associate with many northern temperate terrestrial orchids (Curtis, 1936; Masuhara & Katsuya, 1994; Kulikov & Filippov, 2001). *Rhizoctonia solani* MUCL 47937 was obtained from the Belgian Co-ordinated Collections of Micro-organisms/Mycothèque de l'Université Catholique de Louvain (BCCM/MUCL), Belgium and was isolated from *Brassica oleracea* L. in Belgium. It is a common plant pathogen which also associates with a broad range of orchids (Downie, 1957, 1959; Hadley, 1970; Warcup, 1981; Masuhara & Katsuya, 1994; Otero *et al*., 2002; Sazak & Ozdener, 2006).

The fungal isolates A36, B1 and Q414 were obtained from the Hardy Orchid Society Seed Bank. The A36 fungus was isolated by a member of the Hardy Orchid Society (Alan Dash) and has been found to germinate *Anacamptis spp.*, *Dactylorhiza ssp., Gymnadenia ssp*. and *Spiranthes ssp.* (Alan Leck, personal communication, 2012). The B1 fungus was also isolated by a member of the Hardy Orchid Society (Jim Hill), from the roots of *Dactylorhiza fuchsii* (Druce) Soó, collected in Avon, UK and has been found to germinate *Anacamptis spp., Dactylorhiza ssp., Gymnadenia spp.*, some *Serapias ssp.* and a few other species (Alan Leck, personal communication, 2012). DNA analysis has established it as a type of *Ceratobasidium sp*. from Anastamosis Group C (Heys, 2012). Q414 originated from RGB Kew and is also referred to as F414. It germinates *Anacamptis ssp*. (excluding *Anacamptis pyramidalis* (L.) Rich.), *Dactylorhiza ssp., Serapias ssp.* and *Spiranthes ssp*. (Alan Leck, personal communication, 2012). Further investigation into this strain using the CBS Strain Database has shown that it may be a *Rhizoctonia sp*. CBS 505.93, which was originally isolated from *Dactylorhiza iberica* (M.Bieb. ex Willd.) Soó by T. F. Andersen in 1993 from the Botanical Labratory, University of Copenhagen, Denmark as isolate D347-1. Fungal isolates at RGB Kew are given a number starting with the letter Q (Muir, 1989).

Experimental procedure

All seeds used in the study were mature. Seeds of *A. morio* were obtained in summer of 2013 from plants grown in horticulture in Germany; *D. incarnata* were obtained from the Terrestrial Orchid Forum Seedbank and originated from horticulture in Hungary and were harvested in summer of 2010; seed of *S. bergonii* and *S. parviflora* originated from plants grown in horticulture in Greece, which were harvested in summer of 2013. Seeds were stored in packets made from grease proof paper and were placed in an air tight container with silica gel in a refrigerator until the seeds were sown in December 2013. Fungal cultures were maintained and re-plated on Potato Dextrose Agar, (Formedium, Hunstanton, UK) under aseptic conditions in a laminar flow cabinet (Flowfast V 15P, Faster D:Group, Ferrara, Italy) and stored in a dark temperature controlled cabinet (LMS Series 4 Model 1200, LMS Ltd., Sevenoaks, Kent, UK) at 21±1 ºC prior to use as fungal inoculant.

Before sowing, a triphenyltetrazolium chloride (TTC) seed viability test was performed on seeds of each of the tested orchid species using a method adapted from Dowling & Jusaitis (2012). Seeds were divided amongst seed packets made from circular 90 mm diameter, no. 1 filter paper (Whatman International, Maidstone, Kent, UK), with three replicates of each species and each seed packet containing approximately 50 – 80 seeds. Packets were given an initial 1 minute ultrasonic treatment using a VGT-1000B 40 W, 40 kHz, 750 ml ultrasonic cleaner (GT Sonic, Meizhou City, Guangdong, China) and then placed in 1% w/v NaOCl solution for varying times dependant on recommendations by Malmgren & Nyström (2014) and observation of seed bleaching from previous studies (Hughes, unpublished data); see Table 1 for more information. The packets were then rinsed six times with deionised water before being placed in deionised water for 48 hours at 23 ºC in the dark. After 48 hours, the water was removed and replaced with 1% w/v TTC solution and left for 24 hours at 35 ºC in the dark. After 25 hours, the TTC solution was removed and the seed packets rinsed three times in deionised water to remove excess traces of stain and TTC residues. Seed packets were then unfolded and seeds observed under a stereomicroscope (Leica M275, Leica Microsystems, Milton Keynes, UK). Seeds with visible embryos and viable seeds with embryos that were completely stained red were counted, and mean viability calculated.

The following variables among treatments and within species were standardised: seed sterilisation technique; media volume; vessel size; temperature regime; light levels and photoperiod. Seeds of the studied species were sown on 90 mm petri dishes (Sterilin™, Thermo Fisher Scientific, Newport, UK) containing 30 ml of media each. Symbiotic sowings were performed on modified H1 oat medium (Clements *et al*., 1986, modified by Rasmussen *et al*., 1990a). This media was chosen as it has been used extensively for the symbiotic culture of orchids (Clements *et al*., 1986; Muir, 1989; Rasmussen *et al*., 1990a, 1990b; Wood & Ramsay, 2004). Asymbiotic sowings were placed on Malmgren (1996) modified media (Malmgren & Nyström, 2014), containing 6 g l^{-1} agar, 0.5 g l^{-1} activated charcoal powder, 10 g l^{-1} sucrose, 75 mg l^{-1} MgSO4·7H2O, 75 mg l^{-1} (Ca)3PO4, 75 mg l^{-1} KH2PO4, 5 ml l^{-1} Vaminolact® (Fresenius Kabi, Runcorn, UK), 20 ml l^{-1} pineapple juice (neutralised with 1M NaOH solution), 40 x 1 cm^3 potato pieces per litre (liquidised before adding to unsterilised media). The media was chosen as it has been widely and successfully used in the asymbiotic propagation of orchids (Dutra *et al.*, 2008, 2009; Johnson *et al.*, 2007; Kauth *et al.*, 2005; 2006, 2008b; Kitsaki *et al.,* 2004; Malmgren, 1996; Pierce & Cerabolini, 2011; Pierce *et al.*, 2010, 2015; Stewart & Kane, 2006).

Seeds were sown using the RGB Kew, orchid seed sowing protocol (M. M. Ramsay personal communication, RBG Kew, 2009). Briefly, seeds of each orchid species were portioned into individual packets made from circular 90 mm diameter, no. 1 filter paper, with each seed packet containing approximately 50 – 80 seeds. Fifteen seed packets were used for each species per treatment, giving a total of 90 seed packets per species. Each seed packet was sterilised in 0.5% w/v Sodium dichloroisocyanurate (SDICN) solution for 20 minutes under aseptic conditions in a laminar flow cabinet. Then the seed packets were rinsed sequentially for 30 seconds in two beakers of sterile water. Packets were opened on a sterile 90 mm petri dish and seed from the packet transferred to the respective treatment plate. After the seeds were sown, a small 1 mm^2 piece of fungus was placed at the centre of each germination plate. Upon completion of inoculation with the appropriate fungal strain, all instruments were sterilised in 96% Ethanol before transfer to a glass bead steriliser (Keller Steri 350, Simon Keller AG, Burgdorf, Switzerland) at 250 ºC for 30 seconds. In order to prevent cross-contamination, the surface of the laminar flow cabinet was wiped and

sterilised with 70% Ethanol between inoculations of each fungal treatment. The plates were then sealed using Parafilm M®, labelled and placed in a dark temperature controlled cabinet at 21±1 ºC, as light has been shown to inhibit germination of some terrestrial orchids (Mead & Bulard, 1975) and can result in death of protocorms (Mitchell, 1989).

Dactylorhiza incarnata cultures were incubated for 2 months at 21±1 ºC by which time most germinants had developed up to the point of shoot formation. At this point, protocorms were placed in a dark refrigerator at 2±1 ºC for 2 months, as cold treatments have been shown to promote shoot elongation and leaf formation (Borris, 1970; Hadley, 1970; Beyrle *et al*., 1987; Jørgensen, n.d. in Rasmussen, 2006). As germination of *D. incarnata* was observed on all treatments, an additional control sowing was made with fifteen replicates on H1 media without fungi. Plates were observed for germination and development using a stereomicroscope once every 4 weeks. When protocorms had developed to a stage with elongated shoots, cultures were moved to a plant growth cabinet (Percival AR 66L, Percival Scientific, Perry, IA, USA) with a diurnal temperature cycle of 17:15 ºC (±1 ºC) and a photoperiod of 14:10 h light/dark under Philips Universal T8 cool white fluorescent lights (Philips Electronics UK, Guildford, Surrey, UK) at 35 µmol m^{-2} s^{-1}.

Germination and development was recorded at 8 weeks from the date of sowing. This was done by counting the number and development stage of protocorms on each plate. Protocorm development was scored using the method proposed by Clements *et al*. (1986), see Figure 1. This allowed for determination of germination and development stage. For each treatment, total numbers of germinants at specific development stages and overall germinant totals were recorded for each plate. From this, mean numbers of germinants at each development stage and mean germinant totals were calculated for each treatment along with a standard error of the means. Statistical analysis was performed on data collected at 8 weeks for all species. Firstly, a nominal logistic regression was used to analyse germination on each treatment, using the mean number of protocorms per plate at all stages of development. This was done using JMP version 10.0.2. Secondly, for each species studied, a cumulative link mixed model was performed using development stage as the ordinal response variable, treatment as a fixed factor and replicate added as a random effect. The

model was weighted by number of germinants per stage. This analysis compares the proportion of germinants at different developmental stages across treatments. These analyses were performed using the package “ordinal” (Christensen, 2015), and figures were built with the package “ggplot2” (Wickham, 2009), using the R statistics environment (R Development Core Team, 2015).

Results

Embryo counts revealed the presence of embryos in seeds of all tested species with exception of *A. morio*, where excessive seed bleaching from the seed pre-treatment did not allow for an accurate embryo count. The percentage of seeds with embryos was above 80% in all tested species. Seeds of *S. bergonii* and *S. parviflora* had the highest percentage of embryos at 100%. Viability staining using TTC showed staining in three of the four tested species, with *A. morio* showing no staining. Excessive seed bleaching is likely to be the cause of this result in *A. morio*, as germination was observed in all treatments. The highest staining was observed in *S. bergonii* at 51%, followed by *D. incarnata* at 38%, *S. parviflora* at 30.33%, see Table 1 for details.

Germination for each species was calculated for each treatment using the mean number of protocorms per plate at all stages of development at week 8, (Figure 2; Table 2). The nature of fungal colonisation on many treatment plates did not allow for percentage germination to be calculated as some ungerminated seeds were obscured by fungal growth and their number could not be reliably quantified.

Both orchid species ($\chi^2 = 185.88$, df = 12, p = <0.001) and fungal treatment ($\chi^2 = 464.82$, df = 20, p = <0.001) had a significant effect on the likelihood of germination. There was also a significant effect caused by the interaction between orchid species and fungal isolate ($\chi^2 = 139.19$, df = 28, p = <0.001) suggesting there is differential efficacy of fungi for individual species of orchid. Figure 3 shows the examples of development stages reached at week 8 of germination for all four orchid species on different treatments.

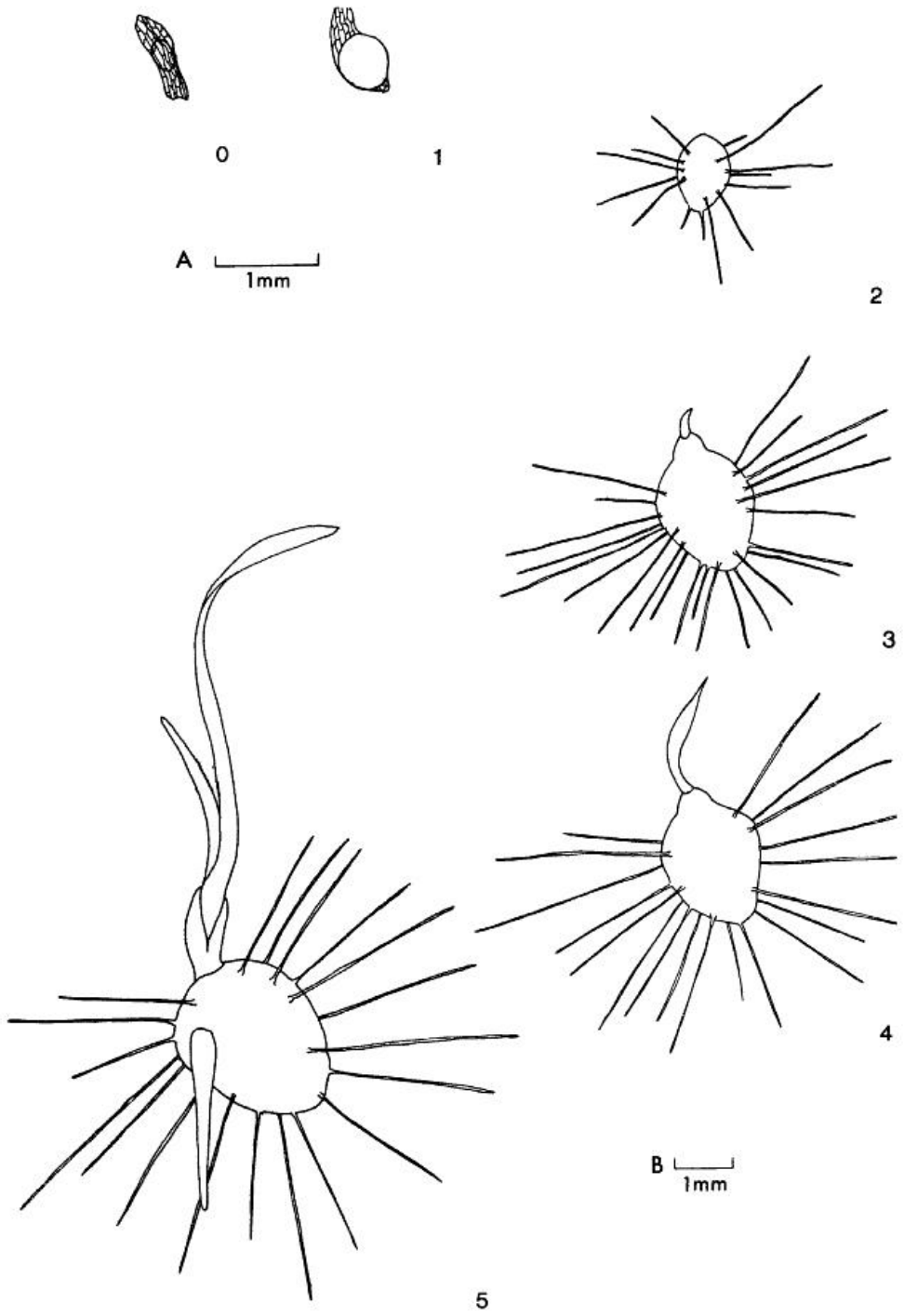

Figure 1. Stages used to assess germination (adapted from Clements *et al*., 1986). 0. Ungerminated seed. 1. Germination of seed with rupture of testa. 2. Production of rhizoids. 3. Production of leaf primordium. 4. Production of first chlorophyllous leaf tissue. 5. Production of root initial. Scale A. Stages 0 and 1. Scale B. Stages 2 to 5.

Table 1. Pre-treatments times prior to TTC staining, mean percentage of seeds with a visible embryos post TTC treatment, mean percentage stained by TTC treatment and highest mean percentage germination with the treatment in parenthesis: B1 (B1 fungus), Rr (*Rhizoctonia repens*) and M (asymbiotic Malmgren media), for *Anacamptis morio, Dactylorhiza incarnata, Serapias bergonii* and *Serapias parviflora.*

Species	1% NaOCl (mins)	Sonication (1 min)	% Visible embryo	% Viability TTC	Optimal % Germination
Anacamptis morio	5	No	-	-	10.21 (Rr)
Dactylorhiza incarnata	15	Yes	89	38	15.47 (B1)
Serapias bergonii	10	Yes	100	51	11.86 (M)
Serapias parviflora	10	Yes	100	30.33	24.2 (M)

Anacamptis morio

Germination was observed on all treatments except with the fungus *R. solani*, (Figure 2; 3). The highest levels of germination were found with the fungus *Rhizoctonia repens* where the mean number of germinants per plate was 10.21±2.19, followed by the asymbiotic Malmgren media (9.21±2.87), B1 (8.93±2.19), A36 (8.8±2.09) with Q414 having the lowest mean number of germinants at 7.2±2.48 (Table 2, Figure 2).

The asymbiotic Malmgren media produced a lower number of germinants at stage 1 (0.64±0.23) and higher mean number of germinants at stage 2 (4.07±1.26) and stage 3 (4.5±1.90), the highest stage of development reached. Although *R. repens* was the best germinator with the highest mean number of germinants per plate at stage 1 (9.71±2.04), this was not followed by a large amount of further development, with 0.5±0.34 reaching stage 2, the highest observed development stage. The fungus Q414 produced the largest mean number of germinants at stage 3 (5.2±2.26), with a lower proportion at stage 2 (1.07±0.33) and stage 4 (0.93±0.46). This orchid species achieved the most advanced development of the studied species, reaching stage 5 with the fungi A36 and B1 after 8 weeks of culture, with both fungi inducing a high proportion

of germinants at stage 3 and 4. The B1 fungus had the highest mean number of germinants at stage 4; 4.73±1.90 and stage 5; 0.33±0.21 (see Table 2, Figure 3a, 3d, 4).

There was a significant effect of treatment on the proportion of germinants at the different development stages. Specifically, compared to the asymbiotic Malmgren media, fungus A36 lead to higher proportion of germinants at later stages (estimate (b) = 2.17, Z = 11.428, p = <0.001) as did the fungus Q414 (b = 1.05, Z = 5.974, p = <0.001) and B1 (b = 2.38, Z = 11.70, p = <0.001), fungus *R. repens* showed a pronounced decrease in the proportion of germinants in later stages (b = -3.1473, Z = -12.726, p = <0.001), see Appendix Table 1, Figure 4.

Dactylorhiza incarnata

Germination was observed on all treatments, except for the H1 symbiotic media control without fungal inoculant. Otherwise, the least amount of germination was observed on asymbiotic Malmgren media with a mean number of germinants per plate of 1.83±0.68, followed by *R. solani*; 4.27±1.37, Q414; 7.8±1.89, A36; 12.36±2.97, *R. repens*; 15.43±5.81 and B1, which had the highest mean number of germinants per plate at 15.47±3.89 (Table 2, Figure 2).

On the asymbiotic Malmgren media *D. incarnata* developed as far as stage 3, with 0.42±0.15 at stage 1, the highest number at stage 2; 1.33±0.59 and a small number at stage 3; 0.08±0.08. *Dactylorhiza incarnata* showed the least amount of development with *R. repens*, only reaching stage 2. However, the mean number of germinants per plate at stage 1; 5.64±2.13 and stage 2; 9.79±4.01 was higher than all other treatments. The other treatments also developed as far as stage 3, the highest stage of development reached. *Rhizoctonia solani* produced a low number of germinants with 1.47±0.52 at stage 1, 1.00±0.59 at stage 2, 1.8±0.55 at stage 3, followed by Q414 with 0.87±0.26 at stage 1, 2.27±0.65 at stage 2, and 4.67±1.16 at stage 3. In comparison A36 had a larger proportion of germinants at stage 1; 2.14±0.83 and stage 2; 5.00±1.56 and a slightly higher number at stage 3; 5.21±1.54. The B1 fungus had 1.71±0.68 at stage 1, 4.87±1.32 at stage 2 and had the highest number of germinants at stage 3; 9.00±2.25, see Table 2, Figure 3c, 5.

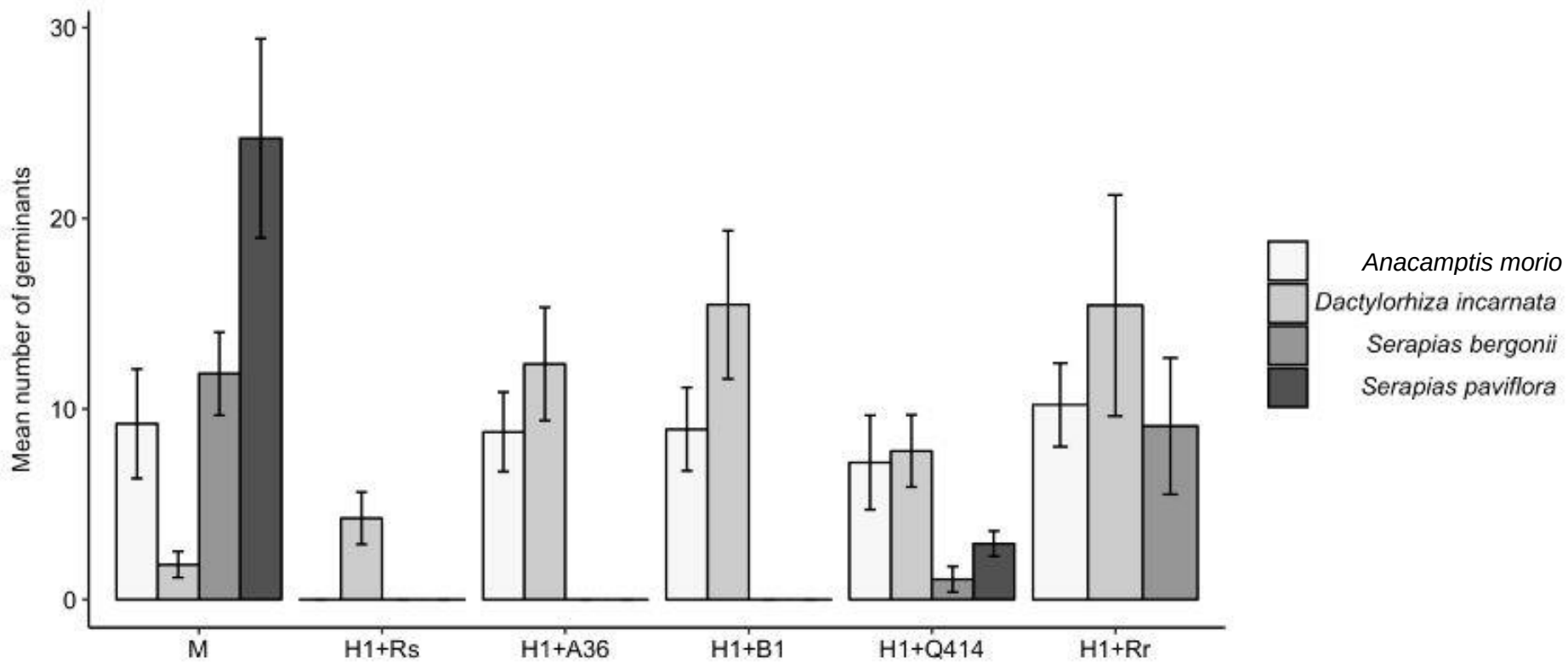

Figure 2. Germination of *Anacamptis morio* (white), *Dactylorhiza incarnata* (light grey), *Serapias bergonii* (grey) and *Serapias parviflora* (dark grey) with the fungi: A36, B1, Q414, *Rhizoctonia repens* (Rr), *Rhizoctonia solani* (Rs) and asymbiotic Malmgren (M) media at 8 weeks from sowing. Values represent mean number of germinants per plate for each treatment. Error bars represent the standard error. Treatment: *Dactylorhiza incarnata* on H1 media was omitted due to lack of germination. Treatment: *Serapias parviflora* with *Rhizoctonia repens* (Rr) was omitted due to contamination.

Table 2. Protocorm development and germination of *Anacamptis morio; Dactylorhiza incarnata; Serapias bergonii; Serapias parviflora* at 8 weeks from sowing on asymbiotic Malmgren; H1 symbiotic media with the fungi A36; B1; Q414; *Rhizoctonia repens; Rhizoctonia repens*. Numbers in parenthesis represent standard errors.

Species	Treatment	Mean % in development stage					Mean % Germination
		Stage 1	Stage 2	Stage 3	Stage 4	Stage 5	
Anacamptis morio	M	0.6 (0.2)	4.1 (1.3)	4.5 (1.9)	0	0	9.2 (2.9)
	H1+A36	0	0.2 (0.1)	5.1 (1.2)	3.2 (0.9)	0.3 (0.2)	8.8 (2.1)
	H1+B1	0.1 (0.1)	0.2 (0.1)	3.6 (0.7)	4.7 (1.9)	0.3 (0.2)	8.9 (2.2)
	H1+Q414	0	1.1 (0.3)	5.2 (2.3)	0.9 (0.5)	0	7.2 (2.5)
	H1+Rr	9.7 (2)	0.5 (0.3)	0	0	0	10.2 (2.2)
Dactylorhiza incarnata	M	0.4 (0.1)	1.3 (0.6)	0.1 (0.1)	0	0	1.8 (0.7)
	H1+A36	2.1 (0.8)	5 (1.6)	5.2 (1.5)	0	0	12.4 (3)
	H1+B1	1.6 (0.7)	4.9 (1.3)	9 (2.2)	0	0	15.5 (3.9)
	H1+Q414	0.9 (0.3)	2.3 (0.7)	4.7 (1.2)	0	0	7.8 (1.9)
	H1+Rr	5.6 (2.1)	9.8 (4)	0	0	0	15.4 (5.8)
	H1+Rs	1.5 (0.5)	1 (0.6)	1.8 (0.6)	0	0	4.3 (1.4)
Serapias bergonii	M	7.2 (1.3)	4.6 (1)	0	0	0	11.9 (2.2)
	H1+Q414	0.1 (0.1)	0.3 (0.3)	0.7 (0.4)	0	0	1.1 (0.7)
	H1+Rr	8.4 (3.4)	0.7 (0.4)	0	0	0	9.1 (3.6)
Serapias parviflora	M	7 (1.3)	17.2 (4.5)	0	0	0	24.2 (5.2)
	H1+Q414	0.5 (0.3)	1.3 (0.3)	1.1 (0.4)	0	0	2.9 (0.7)

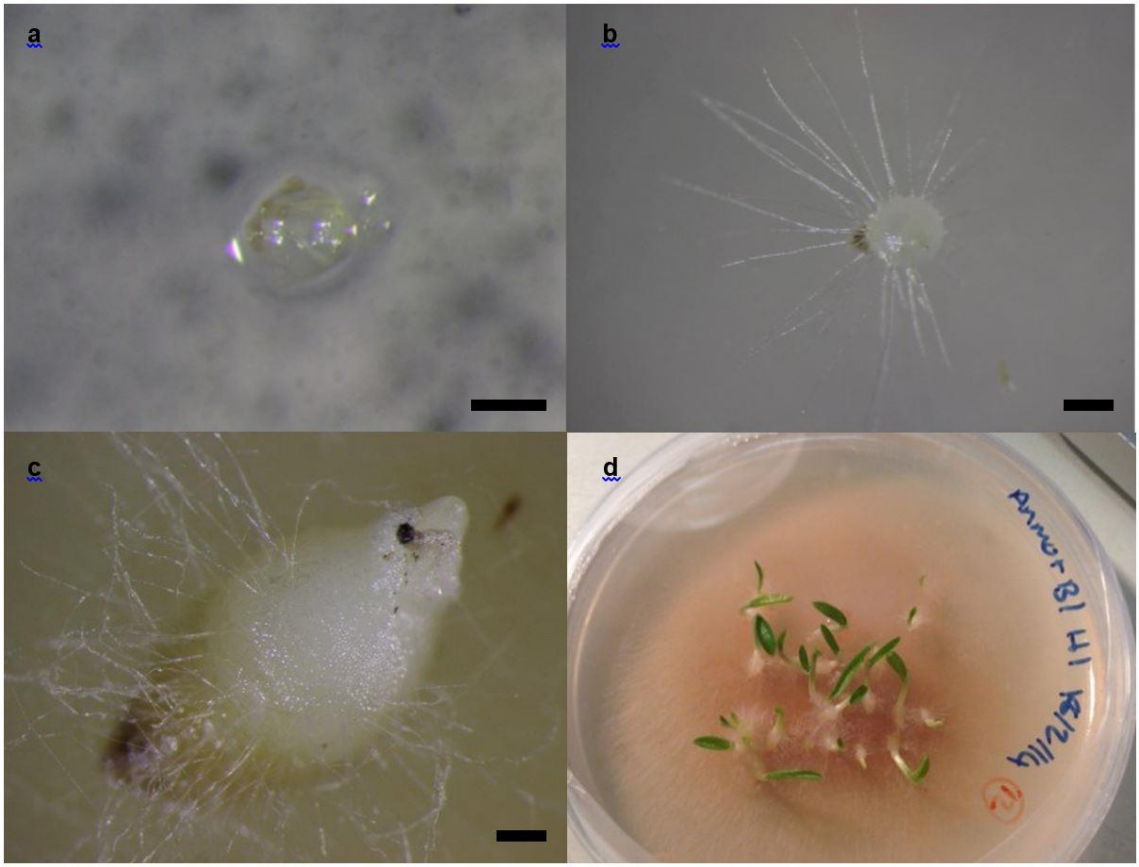

Figure 3. Stages of development at 8 weeks from sowing: **a**) *Anacamptis morio* at stage 1 on Malmgren (M) media, with testa ruptured (scale bar = 0.25 mm). **b**) *Serapias bergonii* at stage 2 on Malmgren media, with production of rhizoids (scale bar = 0.5 mm). **c**) *Dactylorhiza incarnata* at stage 3 with B1 fungus, displaying a leaf primordium (scale bar = 0.5 mm). **d)** *Anacamptis morio* at stage 4 and 5 with B1 fungus, showing chlorophyllous leaf tissue and the start of root production on a 90 mm petri dish.

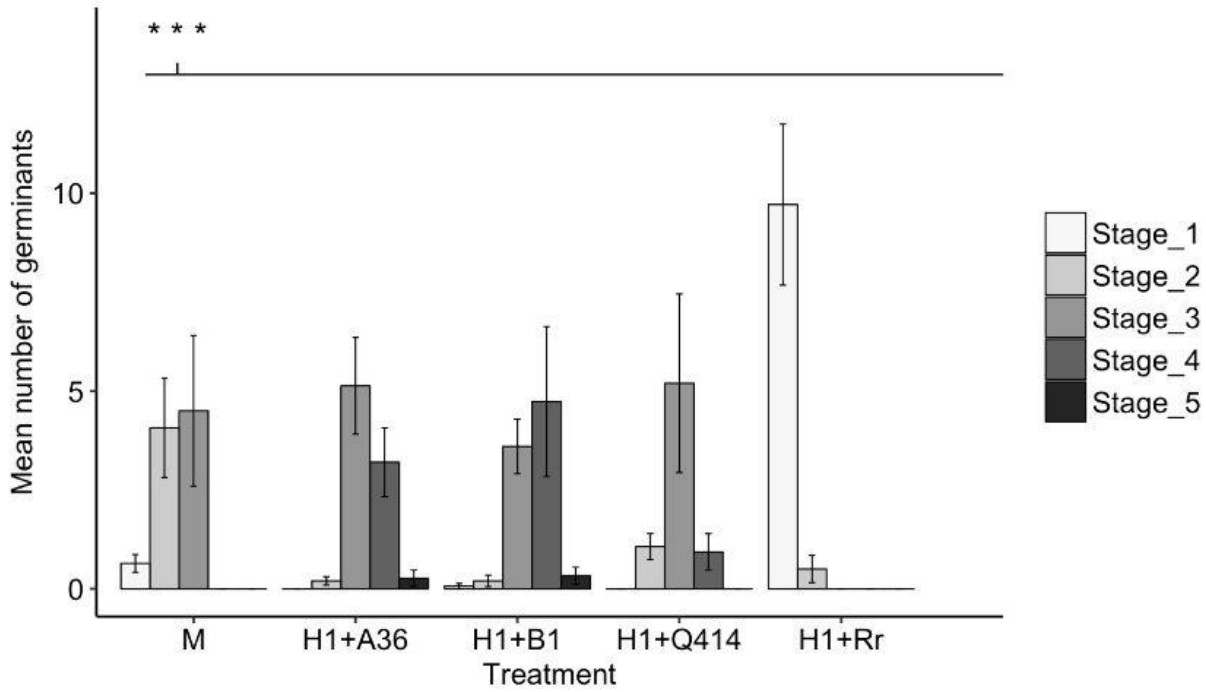

Figure 4. Development of *Anacamptis morio* with the fungi: A36, B1, Q414, *Rhizoctonia repens* (Rr), *Rhizoctonia solani* (Rs) and asymbiotic Malmgren (M) media after 8 weeks from sowing. Development stage 1 (white); stage 2 (light grey); stage 3 (grey); stage 4 (dark grey); stage 5 (black). Values represent mean number of germinants per plate by development stage for each treatment. Error bars represent the standard error. Statistical significant differences were found between the asymbiotic Malmgren media with all symbiotic treatments (*** denotes significant difference of p = <0.001).

There was a significant effect of treatment in *D. incarnata* on the proportion of germinants at different stages. Compared to the asymbiotic Malmgren media, there was no effect of the fungi *R. solani* (b = 0.3228, Z = 1.131, p = 0.25) and a marginally significant negative effect of *R. repens* (b = -0.5299, Z = -1.977, p = 0.048). A positive significant effect of the fungi A36 (b = 0.5478, Z = 2.104, p = 0.035), B1 (b = 0.8715, Z = 3.281, p = <0.001) and Q414 (b = 0.9139, Z = 3.350, p = <0.001) was observed, showing a higher proportion of germinants in later developmental stages (Appendix Table 2), Figure 5).

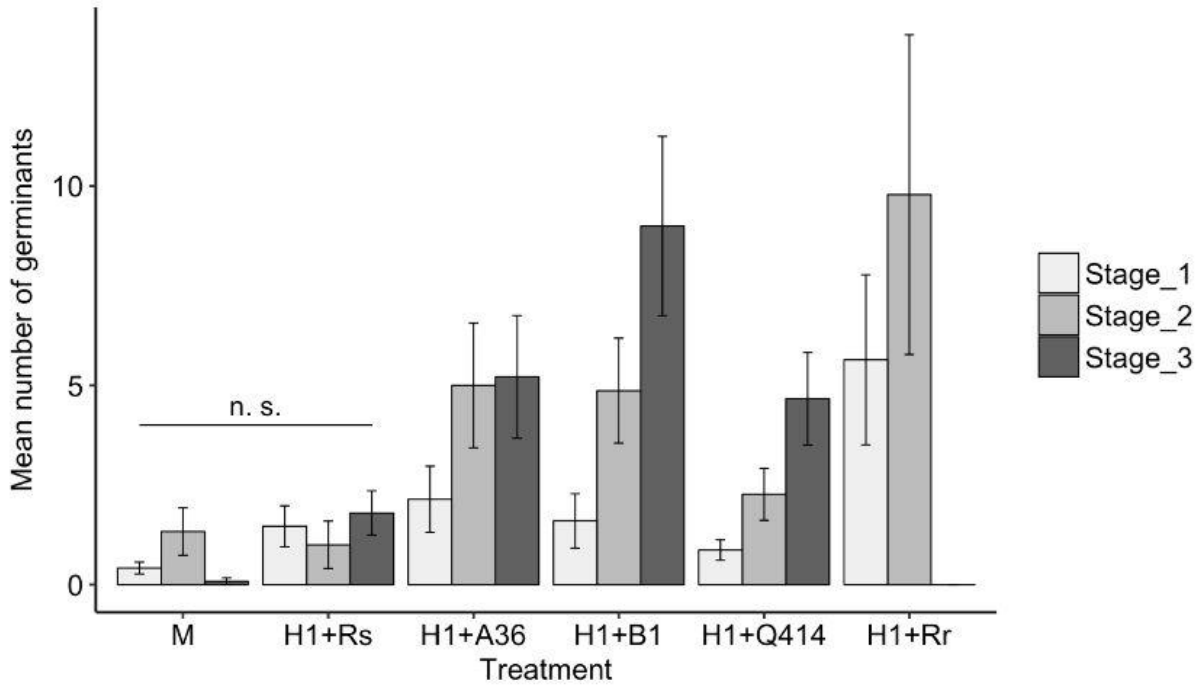

Figure 5. Development of *Dactylorhiza incarnata* with the fungi: A36, B1, Q414, *Rhizoctonia repens* (Rr), *Rhizoctonia solani* (Rs) and asymbiotic Malmgren (M) media after 8 weeks from sowing. Development stage 1 (white); stage 2 (light grey); stage 3 (grey); stage 4 (dark grey); stage 5 (black). Values represent mean number of germinants per plate by development stage for each treatment. Error bars represent the standard error. Statistically significant differences were observed between Malmgren asymbiotic media and all treatments (Appendix Table 2), except between the asymbiotic Malmgren media and H1+Rs (n. s. denotes non-significant values with p = >0.05). Treatment: *Dactylorhiza incarnata* on H1 media was omitted due to lack of germination.

Serapias bergonii

No germination of *S. bergonii* was observed with the fungi; A36, B1 and *R. solani*. A minimal amount of germination occurred with Q414 with a mean number of germinants per plate of 1.07±0.68. A higher proportion germinated with *R. repens*; 9.10±3.58 and the highest amount of germination was achieved with the asymbiotic Malmgren media; 11.86±2.18, see Table 2, Figure 2.

Despite low overall germination, the highest stage of development was observed with Q414, having 0.07±0.07 at stage 1, 0.33±0.27 at stage 2 and 0.67±0.41 at stage 3. *Rhizoctonia repens* had the highest proportion of

germinants at stage1; 8.4±3.38 and a low number at stage 2; 0.70±0.37. The asymbiotic Malmgren media had a high proportion of germinants at stage 1; 7.21±1.35 and stage 2; 4.64±0.96, see Table 2, Figure 3b, 6.

Analyses were performed using three treatments with germinants at the first two developmental stages. When compared to the asymbiotic Malmgren media, there was a highly significant negative effect of *R. repens* in the proportion of seeds at later stages (b = -1.1508, Z = -5.297, p = <0.001) and a marginally positive effect of Q414 (b = 1.2417, Z = 2.014, p = 0.044; Appendix Table 3, Figure 6).

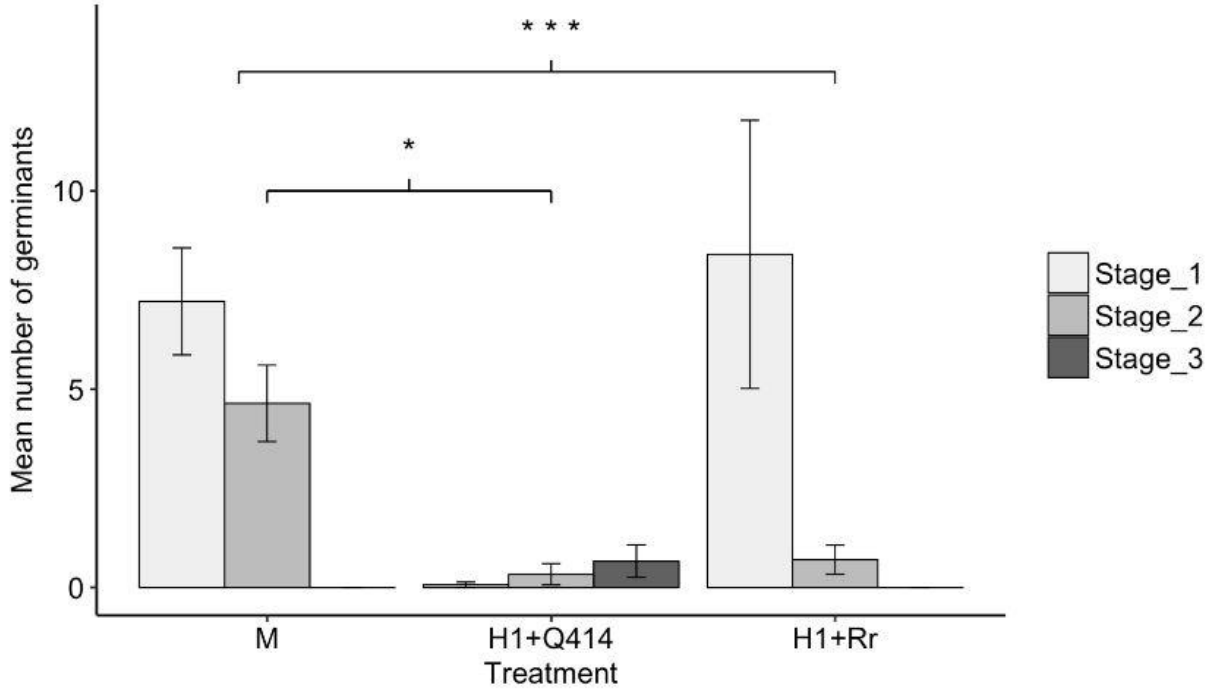

Figure 6. Development of *Serapias bergonii* with the fungi: Q414, *Rhizoctonia repens* (Rr) and asymbiotic Malmgren (M) media after 8 weeks from sowing. Development stage 1 (white); stage 2 (light grey); stage 3 (grey); stage 4 (dark grey); stage 5 (black). Values represent mean number of germinants per plate by development stage for each treatment. Error bars represent the standard error. Asterisks show significant differences between treatments (* denotes significant difference of p = <0.05; ** denotes significant difference of p = <0.01; *** denotes significant difference of p = <0.001).

Serapias parviflora

Similar to *S. bergonii, S. parviflora* showed no signs of germination with A36, B1 and *R. solani*, but in addition no germination was observed with *R. repens*. A large number of plates with *R. repens* suffered from bacterial contamination, which may have affected the results. A similar pattern of germination and development to *S. bergonii* was observed with Q414 and the asymbiotic Malmgren media. Q414 displayed a minimal amount of germination at 2.93±0.67, compared to asymbiotic Malmgren media; 24.2±5.23, see Table 2, Figure 2.

Serapias parviflora showed the highest stage of development with Q414 at stage 3, having 0.53±0.29 at stage 1, 1.33±0.33 at stage 2 and 1.07±0.40 at stage 3. Development for *S. parviflora* differed from *S. bergonii* on asymbitic Malmgren media with 7.00±1.29 at stage 1 and a higher proportion at stage 2; 17.20±4.48, see Table 2, Figure 7.

With *S. parviflora*, analyses could only be performed on the two treatments with germinants and as with *S. bergonii* the first two developmental stages were used. No difference in the proportion of seeds at different developmental stages was found between the two treatments ($b = 0.2316$, $Z = 0.805$, $p = 0.42$; Appendix Table 4, Figure 7).

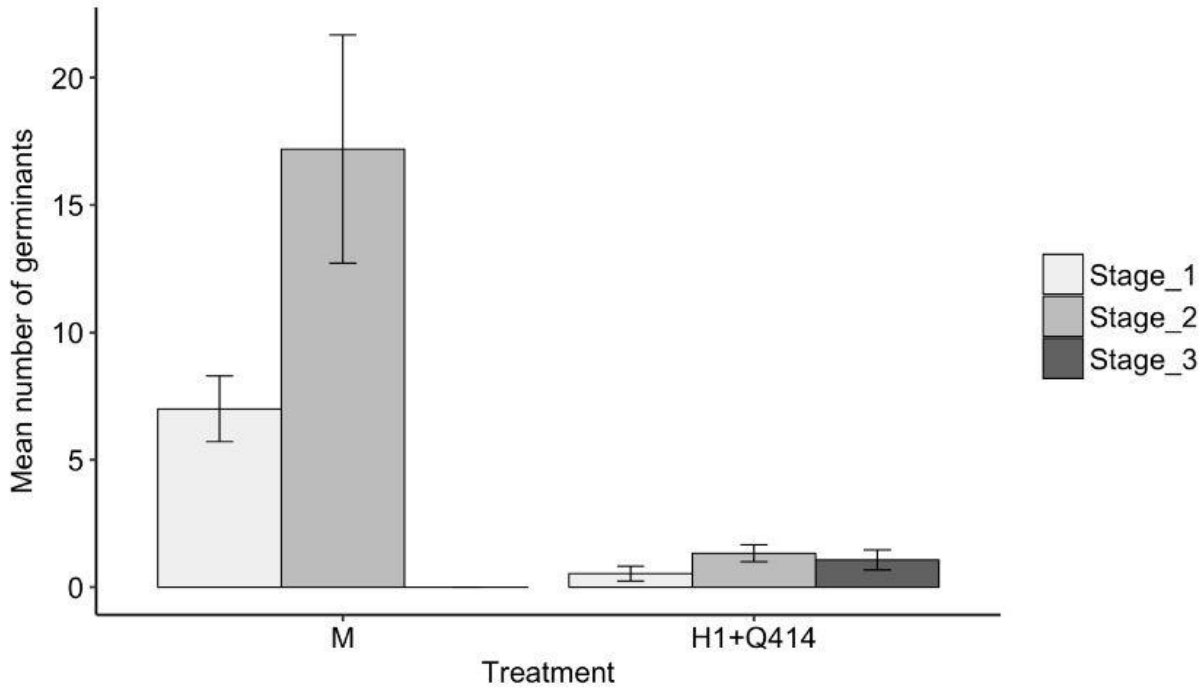

Figure 7. Development of *Serapias parviflora* with the fungi: Q414 and asymbiotic Malmgren (M) media after 8 weeks from sowing. Development stage 1 (white); stage 2 (light grey); stage 3 (grey); stage 4 (dark grey); stage 5 (black). Values represent mean number of germinants per plate by development stage for each treatment. Error bars represent the standard error.

Discussion

The results of this study demonstrate that both orchid species and fungal inoculum had an effect on the germination of Orchidinae species *in vitro* and that the specific combination of plant and fungus was important, suggesting differential efficacy of fungi for individual orchid species.

Species specific responses

Germination in *A. morio* was rapid and displayed low fungal specificity, germinating on all treatments with the exception of *R. solani. Anacamptis morio* reached the most advanced stage of development of all tested species (stage 5), forming chlorophyllous leaf tissue and root initials on the A36 and B1 fungal strains. The low fungal specificity observed in *A. morio* reflects observations made by Muir (1989), where a number of *Anacamptis* species were able to germinate with a broad range of fungi isolated from European tuberous

terrestrial orchids. Germination of *A. morio* was highest with *R. repens* and on the asymbiotic Malmgren media. The utilised strain of *R. repens* was isolated from *A. morio* in 1934 and considering that this isolate has been in culture for 80 years it is noteworthy that it is still a capable germinator of its original host species. The high initial germination of *A. morio* with *R. repens* was not followed by a high level of subsequent development, with protocorms only reaching stage 2 after 8 weeks of culture. This suggests that other fungi may be required to induce further development. Although similar observations have been made in the field with rhizomatous terrestrial orchids (Bidartondo & Read, 2008), this may be the first time the possibility has been reported for a Eurasian tuberous species. The fungi A36, B1 and Q414 resulted in the furthest development of *A. morio*, with germinants reaching stage 4 with all three fungi and stage 5 with A36 and B1. The B1 fungus had the highest proportion of germinants at stage 5 after 8 weeks of culture and can be considered to be the most effective treatment overall.

Dactylorhiza incarnata germinated on all treatments developing as far as stage 3 with the fungal strains A36, B1 and Q414. A subsequent control experiment sowing *D. incarnata* on uninoculated modified H1 oat medium did not result in germination after 8 weeks of culture. This indicates that on this media germination was highly likely to be fungal induced and that *D. incarnata* is unspecific in its choice of fungal partners. These results concur with similar fungal compatibility experiments conducted with *D. fuchsii* (Salman *et al*., 2002), *D. majalis* (Rchb.) P. F. Hunt and Summerh. (Rasmussen, 2006) and *D. purpurella* (T. Stephenson and T. A. Stephenson) Soó (Hadley, 1970). The B1 fungus resulted in the highest number of germinants, closely followed by *R. repens*. A low number of germinants were observed with *R. solani* and the fewest on the asymbiotc Malmgren media. The B1 fungus was the most effective treatment, in terms of inducing development, producing the largest number of germinants at stage 3. This fungus was originally isolated from *D. fuchsii* (Alan Leck, personal communication, 2012) which may account for its success. The least amount of development was obtained with *R. repens* showing a similar pattern to that observed in *A. morio*, although with *D. incarnata*, there were a higher proportion of germinants at stage 2 rather than stage 1. The low germination observed on asymbiotic Malamgren media may

have been the result of insufficient scarification during the sterilisation process. Asymbiotic sowings are more dependent on the action of sterilants rather than fungi to weaken the seed coat and aid germination (Rasmussen, 2006). A large number of *D. incarnata* seeds remained relatively dark in colour after the 20 minute 0.5% SDICN treatment which is far from the optimal cream colour (Malmgren & Nyström, 2014), suggesting a longer treatment time may have provided better results.

Serapias bergonii and *S. parviflora* displayed a far greater fungal specificity compared to *A. morio* and *D. incarnata*. Whilst both germinated most prolifically on the asymbiotic Malmgren media, *S. bergonii* was only found to germinate with *R. repens* and Q414, while *S. parviflora* germinated only with Q414. *Serapias bergonii* produced a relatively high number of germinants with *R. repens*, however as observed in *A. morio* and *D. incarnata*, development only reached as far as stage 2, and as found for *A. morio*, a higher proportion of germinants were at stage 1 compared to stage 2 after 8 weeks of culture. Unfortunately, a large amount of bacterial contamination affected many plates of *R. repens* with *S. parviflora*, preventing the collection of sufficient data. A repeat sowing was considered, but there were insufficient seeds to produce the desired number of replicates. Both *S. bergonii* and *S. parviflora* displayed comparatively low germination with Q414, however development was more advanced; reaching stage 3 compared with stage 2 on asymbiotic Malmgren media. The ability of the fungal strain Q414 to act as a germinator of *Serapias* concurs with reports from members of the Hardy Orchid Society (Alan Leck, personal communication, 2012). It is likely that given more time, symbiotic cultures of *S. bergonii* and *S. parviflora* with Q414 may have reached stage 4 and 5. Data from Clements *et al*., (1986) suggests that it can take around 92 days for germinants to reach these stages. Despite slower development, the asymbiotic method can be considered the best method trialled here in terms of overall numbers of germinants produced.

Experimental variables

Development on asymbiotic Malmgren media was more retarded when compared with the most effective symbiotic treatments for all orchid species tested. Many terrestrial orchids have been found to develop only to a certain

stage on some asymbiotic media, which could help explain the slower development seen here (Muir, 1989; Malmgren & Nyström, 2014). Malmgren & Nyström (2014) state that once protocorms of various tuberous terrestrial orchids have reached 2 – 5 mm in size, growth on asymbiotic media stops and re-plating to fresh media is required. Re-plating germinants onto fresh media or to media with a different formulation containing a higher sucrose content and/or different complex organic ingredients can remedy this issue (Rasmussen, 2006; Pierce *et al.*, 2015). Exposing germinants of temperate and Mediterranean terrestrial orchids to periods of cool winter temperatures at 4 – 8 ºC after re-plating has also been suggested to induce leaf, root and tuber formation (Malmgren & Nyström, 2014). These factors will be implemented with further culture of these species.

In this study, TTC staining gave higher seed viability than when compared to germination on optimal asymbiotic or symbiotic treatments (Table 1). Other investigators have obtained mixed results when comparing seed viability testing and asymbiotic germination. Similar to this study, Van Waes & Debergh, (1986) achieved higher TTC seed viability compared to asymbiotic treatments in European terrestrial orchids. Though in some species optimal asymbiotic treatments gave similar germination values to TTC viability. Dowling & Jusaitis, (2012), however, obtained higher germination on most asymbiotic media when compared to TTC viability in Australian terrestrial orchids. It is evident that TTC viability testing can provide an indication of seed viability but more work remains to be done to optimise these methods. The main issue facing this technique and similarly the pre-treatment of seed prior to sowing is the variability of the orchid seed coat. Orchid seed of different genera, species and geographic origin have differential permeability of the seed coat (Van Waes & Debergh, 1986, Kauth *et al*., 2011). This can have an effect on any seed pre-treatment and in turn the ability of the stain to interact with the embryo, potentially giving erroneous results as observed in *A. morio.* Here the seed pre-treatment with 1% w/v NaOCl proved to be too strong or lengthy for the seed of this species, likely killing the embryos and preventing activation of the stain. The subsequent sowings with the standardised treatment using 0.5% SDICN did prove more suitable with germination on all bar one treatment.

Determining an appropriate seed pre-treatment can be challenging as protocols for effective staining and germination vary. Initially a protocol, pre-

treating seeds by soaking in sucrose solution prior to staining (Hosomi *et al.*, 2011; 2012) was trialled, but was found to be ineffective. Other investigators; (Van Waes & Debergh, 1986; Dowling & Jusaitis, 2012) have utilised bleach pre-treatment of terrestrial orchid seed before staining, treating seeds for the same periods of time as were used for seed sowings. Both studies, however, differ in their use of bleaching agents and bleaching times. In the case of Van Waes & Debergh, (1986), a number of sterilisation times were trialled in order to obtain optimal seed sterilisation times. Utilising a sterilisation technique as outlined by Ponert *et al*., (2011), (See Chapters: 4; 5; 6) which allows for close observation of seed during the sterilisation process could have helped ascertain more effective treatment strengths and times.

Another factor to consider in determining viability of orchid seed is seed dormancy, as even when visible embryo counts and TTC staining indicate optimal viability; germination does not occur (Whigham *et al*., 2006, Hughes, unpublished data). It is unknown whether any orchid seed experiences long term dormancy, however morpho-physiological dormancy does occur (Baskin & Baskin, 1998; Rasmussen, 2006). Seed dormancy can be overcome by chemical and mechanical breakdown of the testa and signals from appropriate fungal partners, as well as abiotic factors such as lengthy imbibition, temperature regime, darkness, atmospheric composition and chemical signalling. Such mechanisms can also be broken by investigators through seed scarification processes, associations with suitable fungi, harvesting immature seed and inclusion of certain chemicals and phytohormones in the culture media (Rasmussen, 2006). As a result of the multitude of factors effecting orchid seed germination, the most effective germination methods for many species remain unknown and implementing a standardised method can prove problematic. Optimising these factors on a species or preferably, individual basis is vital to ensure the best germination is obtained in temperate terrestrial orchids.

Fungal specificity and compatibility

The results of this study add further credence to the notion that fungal specificity can vary between genera and species, where some orchids exhibit high levels of specificity and others are more generalist, germinating with a variety of fungi. Fungal reliance can vary considerably among genera, species

and intra-species due to different habitat factors (Rasmussen, 2006; Roberts & Kingsley, 2008). A fungal compatibility trial conducted by Muir (1989) produced varied germination and development responses among Eurasian tuberous terrestrial species. Certain fungal isolates, obtained from the tested species, proved to be excellent germinators with a variety of the tested species. Others stimulated further growth and development and some provided no benefit at all. Muir's (1989) work also demonstrated the propensity for *Serapias spp*. to associate with certain fungal strains and concurs with the more strict fungal specificity observed with *Serapias spp.* in this study.

My observations of *R. repens* as a germinator indicate successful initial interactions across the study species, but further development of protocorms did not occur in any species despite the fact the fungus was isolated from plants of *A. morio*. Such results may support the notion of fungal bottlenecks in protocorm development (Bidartondo & Read, 2008). Bidartondo & Read (2008) showed that initial protocorms and mature plants of Eurasian rhizomatous terrestrial species had broad associations with a number of fungi. Later protocorm stages, however, showed increasing specificity with many protocorms potentially requiring interactions with additional fungal species to induce further development post germination. Observations of increasing fungal specificity over time have been made with two tuberous terrestrial ''weed-like (disturbance tolerant, rapidly spreading orchids)', the Australian; *Microtis media* R.Br. (Bonnardeaux *et al.*, 2007, Hughes, Chapter 2) and the invasive South African tuberous terrestrial orchid; *Disa bracteata* Sw. (Bonnardeaux *et al.*, 2007). Both species initially formed protocorms with a broad range of fungi but those that initiated later seedling development were more restricted. The results of this study and those of Hadley, (1970); Salman *et al*., (2002); Rasmussen, (2006) have shown that *Dactylorhiza spp.* can associate with a broad web of fungi. These and the findings of Bonnardeaux *et al.*, (2007) may help to explain the relative abundance, broad distribution and propensity for colonizing disturbed sites by several species within this genus.

When interpreting these results it should also be noted that studies by Masuhara & Katsuya, (1994) have demonstrated that fungal specificity *in vitro* may be lower than in natural habitats. Further experiments using *in situ* seed baiting techniques with the studied species, followed by subsequent fungal

isolation and identification as demonstrated by Bidartondo & Read (2008) could help confirm whether this is true for the study species. Reintroducing asymbiotically propagated terrestrial orchids without appropriate fungal partners has in some cases such as *Dactylorhiza hatagirea* (D.Don) Soó proven unsuccessful (Vij *et al*., 1995). However, reintroductions of plants originating from symbiotic culture with fungi isolated from the same or similar species have proved to be more successful (Stewart *et al*., 2003; Aggarwal & Zettler, 2010). Similarly; Muir (1989); Anderson, (1991) and Wood & Ramsay (2004) were successful in introducing symbiotically propagated seedlings into semi-natural areas. Thus gaining a more detailed understanding of fungal specificity and compatibility will be of particular importance to the future reintroduction of terrestrial orchids through integrated conservation programmes as detailed by Swarts (2007); Aggarwal & Zettler, (2010).

With the need to conserve many terrestrial orchids becoming ever more urgent and an increasing horticultural demand, it is vital that effective propagation techniques are developed. This study has demonstrated advantageous combinations of plants and fungi that could be utilised in future propagation of Eurasian tuberous terrestrial orchids. Continuing the investigation to acquire data on long term culture and *ex vitro* establishment will help gain a better understanding of the effectiveness of treatments and allow for appropriate culture protocols to be established. Further experiments on fungal compatibility can help validate current theories on orchid-fungal specificity, potentially revealing important relationships between orchids and fungal genera, and identifying viable fungal symbionts for orchid propagation.

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Appendix

Appendix Table 1. Cumulative link mixed model of the proportion of seeds at different stages of development in *Anacamptis morio*. Asymbiotic Malmgren media is used as reference.

	Estimate	**Standard Error**	**Z**	***P***
Treatment (H1+A36)	2.1746	0.1925	11.297	<0.001 ***
Treatment (H1+B1)	2.3845	0.2013	11.845	<0.001 ***
Treatment (H1+Q414)	1.0551	0.1714	6.155	<0.001 ***
Treatment (H1+Rr)	-3.1473	0.2473	-12.726	<0.001 ***

Appendix Table 2. Cumulative link mixed model of the proportion of seeds at different stages of development in *Dactylorhiza incarnata*. Asymbiotic Malmgren media is used as reference.

	Estimate	**Standard Error**	**Z**	***P***
Treatment (H1+A36)	0.5478	0.2604	2.104	0.0353 *
Treatment (H1+B1)	0.8715	0.2656	3.281	0.001 **
Treatment (H1+Q414)	0.9139	0.2728	3.350	<0.001 ***
Treatment (H1+Rr)	-0.5299	0.2680	-1.977	0.0480 *
Treatment (H1+Rs)	0.3228	0.2853	1.131	0.2579

Appendix Table 3. Cumulative link mixed model of the proportion of seeds at different stages of development in *Serapias bergonii.* Asymbiotic Malmgren media is used as reference.

	Estimate	**Standard Error**	**Z**	***P***
Treatment (H1+Q414)	1.2427	0.6169	2.014	0.044 *
Treatment (H1+Rr)	-1.1508	0.2172	-5.297	<0.001 ***

Note: Only the first two stages of development were compared with this species

Appendix Table 4. Cumulative link mixed model of the proportion of seeds at different stages of development in *Serapias parviflora.* Asymbiotic Malmgren media is used as reference.

	Estimate	**Standard Error**	**Z**	***P***
Treatment (H1+Q414)	0.2316	0.2877	0.805	0.421

Note: Only the first two stages of development were compared with this species.

Chapter 4.

Symbiotic culture enhances *in vitro* germination and development of two European terrestrial orchids; *Anacamptis laxiflora* and *Ophrys apifera*

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In review

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Abstract

Optimising propagation methods that assist in the production of viable plants for horticulture and conservation can help to remove collection pressures on wild populations and aid efforts to conserve rare species. An experimental approach was used to refine culturing conditions for two species that have potential commercial importance. To ascertain the most effective *in vitro* propagation method, seeds of two European terrestrial orchid species: *Anacamptis laxiflora* (Lam.) R. M. Bateman, Pridgeon & M. W. Chase and *Ophrys apifera* Huds. were sown in asymbiotic Malmgren modified media and symbiotic H1 media with the B1 fungal isolate (*Ceratobasidium sp*. Anastamosis Group C) for *A. laxifolia* and the unidentified FOA8 fungal isolate for *O. apifera*. Germination and development were recorded at 5 and 8 weeks from sowing.

A significantly higher number of *A. laxiflora* germinants were obtained with the symbiotic method, with around three times more germinants than the asymbiotic method. Germination in *O. apifera* was far lower when compared to *A. laxiflora*. The highest overall germination was achieved with symbiotic culture, with almost double the relative number of germinants developing compared to the asymbiotic method. However, symbiotic germination in *O. apifera* was much more variable across plates, compared with asymbiotic germination. Development in both orchids progressed to a more advanced state in symbiotic cultures with *A. laxifolia* producing leaves and roots and *O. apifera* forming protocorms with rhizoids. Development of *O. apifera* largely arrested after 3 months on both treatments with asymbiotic germinants developing only as far as stage 1 and symbiotic germinants as far as stage 2. At 18 months of culture, 111 symbiotic *A. laxiflora* plantlets and 47 asymbiotic plantlets were de-flasked. After 30 months culture, 12 months of which were *ex vitro*, no asymbiotic ex-plants had survived, however, around 80 symbiotic ex-plants survived with some asexual propagation taking place amongst larger plants. At 36 months culture the first flower bud was observed.

Keywords Orchidaceae • Germination • Terrestrial • Mycorrhizae • Conservation

Introduction

Terrestrial orchids are widely regarded as being one of the most vulnerable higher plant groups (Zettler, 1997b). This is largely due to the complex nature of orchid reproduction, germination and establishment, the most important of which are highly specific pollinators and symbiotic fungal partners. These specialisms can result in species with narrow geographic ranges and distributions limited to certain habitats (Cribb *et al*., 2003). As a result, orchid populations can be easily impacted by anthropogenic effects such as habitat loss, overexploitation and climate change (Swarts & Dixon, 2009a). In cases where populations have become so small and fragmented that they are unviable, there is a strong case for *ex situ* conservation efforts. For orchids, this requires replication or substitution of the environmental conditions and symbiotic fungal partners needed for germination and growth.

While many plant species form associations with mycorrhizal fungi, orchids have taken the relationship one step further. Their specialisation has resulted in a heavy reliance on mycorrhizal fungi for at least some stages in their life cycle, most notably during germination. However, as Rasmussen (2006) points out, despite a vast literature addressing the propagation of orchids, the use of symbiotic methods remains understudied. Improved isolation and identification of appropriate orchid fungal symbionts may also have commercial implications. Rännbäck (2007) highlights the increasing horticultural interest in terrestrial orchids and suggests that the growing commercial demand for plants makes orchid propagation a key area for further research, particularly concerning orchid-mycorrhizal interactions.

Terrestrial orchids are most commonly propagated *ex situ* from seed using agar based nutrient media. Propagation can be symbiotic, where a mycorrhizal fungus is cultured in association with orchid seed, or asymbiotic, where seeds are sown without fungi on a sterile nutrient media (Rasmussen, 2006). Both methods have been used with varying degrees of success to propagate and conserve rare orchid species *ex situ* (Anderson, 1991; Stewart *et al*., 2003; Aggarwal & Zettler, 2010; Pierce *et al.*, 2010, 2015). Asymbiotic media have been widely and successfully used for commercial propagation purposes, whereas symbiotic propagation methods are largely neglected for commercial

use and have mostly been used by amateur hobbyists and conservation practitioners (Zettler, 1997b; Kauth *et al*., 2008a; Seaton *et al.,* 2011). There is much debate as to which is the 'best' method of propagation. Seaton *et al.* (2011) state that when combined with the appropriate fungi, some terrestrial orchids can be grown much faster with higher rates of survival *ex vitro* than when grown using comparable asymbiotic methods. In contrast where suitable asymbiotic media are found, large numbers of mature plants can be produced efficiently, without the effort of isolating suitable fungi and providing the correct conditions for fungal symbiosis. Scientific comparisons of the two methods are rare and where they have been undertaken, results are inconclusive (Muir, 1989; Rasmussen *et al*., 1990a; Anderson, 1991; Johnson *et al*., 2007; Otero & Bayman, 2009).

For propagation using asymbiotic methods, the nutrients that mycorrhizal fungi would normally provide are instead included in the media. In its simplest form, asymbiotic media is a mixture of sugars and fertiliser solution with the addition of a gelling agent. The same basic nutrients that are needed for all plant growth, such as Nitrogen (N), Phosphorus (P), Potassium (K) and Magnesium (Mg), are provided, along with varying amounts of trace elements, amino acids, vitamins, phytochemicals and other complex organic substances (Seaton *et al.* 2011). There are a great number of different formulations available (Rasmussen, 2006), however many terrestrial orchid genera and species only germinate on a narrow range of asymbiotic media (Seaton *et al.* 2011).

Using symbiotic methods developed in the 1970s and 1980s by Warcup, (1971), Clements & Ellyard (1979) and Clements (1982a), a number of Australian, European and North American terrestrial orchids have been successfully propagated (Clements 1982a, Clements *et al*., 1986; Muir, 1989, Anderson, 1991, Stewart *et al*., 2003, Batty *et al*., 2006a, 2006b; Bonnardeaux *et al.,* 2007). Media for symbiotic germination differs from asymbiotic media by the inclusion of polysaccarhides such as cellulose (Hadley, 1969) and starch, for example oatmeal (Clements & Ellyard, 1979; Clements, 1982a; Rasmussen, 2006). These carbohydrates cannot be utilised directly by the orchid seedlings, but are made accessible through fungal metabolism (Rasmussen, 2006). Previous studies using symbiotic methods have used a variety of fungal isolates (Warcup, 1971; Clements 1982a, Clements *et al*., 1986; Muir, 1989, Anderson, 1991, Stewart *et al*., 2003). These have been partly determined by the differing requirements of

the orchid species utilised, with fungal specificity during germination and development being a key factor.

Associations between orchids and fungi can be generalist, with orchids associating with several different fungal genera (Warcup, 1981, 1985; Kulikov & Filippov, 2001; Athipunyakom *et al.*, 2004; Bonnardeaux *et al.*, 2007; Bidartondo & Read, 2008; Yamato & Iwase, 2008), or specialist, where the orchids only associate with one or a group of closely related fungal species (Warcup, 1971, 1981, 1985; Kulikov & Filippov, 2001; Athipunyakom *et al.*, 2004; Leake, 2004; Bonnardeaux *et al.*, 2007; Yagame *et al.*, 2007). In addition, whilst orchid seed germination can occur with numerous fungal species, further development can often only be supported by one or a small number of fungi (Bidartondo & Read, 2008, Chapter 2). Orchidaceous mycorrhizal fungi, e.g. *Ceratobasidium, Thanatephorus, Tulasnella spp*. and their corresponding asexual anamorphs, have been found to be suitable orchid symbionts *in vitro* (Harvais & Hadley, 1967; Hadley, 1970; Warcup, 1973; Clements *et al*., 1986). Previous studies have shown that such fungi isolated from both orchid roots and non-orchid sources can stimulate germination in seeds of similar (Harvais & Hadley, 1967; Muir, 1989) and more distantly related orchid genera (Hadley, 1970).

Studies utilising symbiotic methods have largely been as a result of scientific investigations and conservation initiatives (Warcup, 1971; Clements 1982a, Clements *et al*., 1986; Muir, 1989, Anderson, 1991, Stewart *et al*., 2003; Aggarwal & Zettler, 2010), with few studies giving consideration to commercial propagation (Batty *et al*., 2006a; Liu *et al*., 2010; Aewsakul *et al*., 2013). The time and effort required for extraction, isolation, testing and maintenance of pure cultures of mycorrhizal fungi is a complicated process, as a result, symbiotic seed germination is likely to be more expensive than asymbiotic techniques (Rasmussen, 2006). However, these additional efforts may be discounted by increased survival and associated benefits of fungal symbiosis *ex vitro* (Anderson, 1991; Batty *et al*., 2006a; Aggarwal & Zettler, 2010; Liu *et al*., 2010; Aewsakul *et al*., 2013). Work still remains to be done on the effectiveness of various symbiotic approaches with a large number of orchid genera. In particular, these methods may still prove to be effective at promoting the germination of seeds and the development of mature plants for some of the more troublesome

genera and species, where asymbiotic methods have proven to be unsuccessful (Rännbäck, 2007).

The aim of this study was to test the hypothesis that symbiotic propagation would be more effective than asymbiotic propagation at inducing germination and facilitating development in two common European tuberous terrestrial orchids; Lax-flowered or Jersey Orchid (*Anacamptis laxiflora* (Lam.) R.M. Bateman, Pridgeon & M.W. Chase), and the Bee Orchid (*Ophrys apifera* Huds.). Both species have potential horticultural value, suitable fungal symbionts are available and they can both act as models for rarer species within their genera. The objectives were to compare the most common symbiotic and asymbiotic method for germination and production of viable plants for horticulture and conservation, with the intention that improved propagation methods will help to remove pressures on wild populations.

Materials and methods

Mature seeds of *Anacamptis laxiflora* were obtained from the Balkan Rare Plant Nursery (Elena, Veliko Tarnovo, Bulgaria) from plants grown in horticulture and harvested in the summer of 2013. Mature seeds of *Ophrys apifera* were obtained from Ted Weeks (Hardy Orchid Society member) and were harvested in the summer of 2010 from plants growing on an industrial site in Avon, UK (with the land owner's permission). Seeds were stored in packets made from greaseproof paper and were placed in an air-tight container with silica gel at 3±1 ºC until use. The symbiotic fungus B1 was obtained from the Hardy Orchid Society Seed Bank (UK) for use with *A. laxiflora*. The B1 fungus was isolated by a member of the Hardy Orchid Society (Jim Hill), from the root of *Dactylorhiza fuchsii* (Druce) Soó*,* collected in Avon, UK. This isolate was chosen because it has been shown to be effective at germinating *Anacamptis ssp.* (Alan Leck, personal communication, 2012). DNA analysis in 2011 established it as a type of *Ceratobasidium sp*. from Anastamosis Group C (Heys, 2012). The unidentified isolate FOA8, chosen for symbiotic sowings of *O. apifera*, was isolated and provided by a member of the Hardy Orchid Society (Ted Weeks). The fungus was originally isolated from protocorms of *O. apifera* found at the same site as the parent plants used in this

study. FOA8 has been reported to induce germination and development in *O. apifera* and a number of its subspecies (Ted Weeks, personal communication, 2013). Fungal cultures were sub-cultured on Potato Dextrose Agar, (Formedium, Hunstanton, UK) under aseptic conditions in a laminar flow cabinet (Flowfast V 15P, Faster D:Group, Ferrara, Italy) and stored in a dark temperature controlled cabinet (LMS Series 4 Model 1200, LMS Ltd., Sevenoaks, Kent, UK) at 21±1 ºC prior to use as fungal inoculant.

The following factors among treatments and within species were standardised: seed sterilisation technique; media volume; vessel size; temperature regime and light levels/periods. Seeds of *A. laxiflora* were sown in February 2014 and seeds of *O. apifera* were sown in March, 2014 on 90 mm sterile petri dishes (Sterilin™, Thermo Fisher Scientific, Newport, UK) containing 30 ml media and sealed with Parafilm M® (Bemis, Neenah, WI, USA). Asymbiotic sowings of *A. laxiflora* were placed on Malmgren (1996) modified media (Malmgren & Nyström, 2014), containing 6 g l^{-1} agar, 0.5 g l^{-1} activated charcoal powder, 10 g l^{-1} sucrose, 75 mg l^{-1} MgSO4·7H2O, 75 mg l^{-1} (Ca)3PO4, 75 mg l^{-1} KH2PO4, 5 ml l^{-1} Vaminolact® (Fresenius Kabi, Runcorn, UK), 20 ml l^{-1} pineapple juice (neutralised with 1M NaOH solution), 40 x 1 cm^3 potato pieces per litre (liquidised before adding to unsterilised media). The same media formulation was used for asymbiotic sowings of *O. apifera*, except that pineapple juice was omitted and the potato was replaced with equivalent sized turnip pieces as recommended by Malmgren & Nyström (2014). This media was chosen as it has been widely and successfully used in the asymbiotic propagation of orchids (e.g. Kitsaki *et al.,* 2004; Malmgren, 1996; Pierce & Cerabolini, 2011; Pierce *et al.*, 2010, 2015). Symbiotic sowings of both species were performed on modified H1 Oat Medium (Clements *et al*., 1986, modified by Rasmussen *et al*., 1990a). This media was chosen as it has been used extensively for the symbiotic culture of orchids (e.g. Clements *et al*., 1986; Muir, 1989; Rasmussen *et al*., 1990a, 1990b; Wood & Ramsay, 2004).

Seed sterilisation was undertaken in a laminar flow cabinet using the sterile syringe and mesh method (Ponert *et al*. 2011) with 48 micron nylon mesh (Plastok, Birkenhead, UK) and an initial 70% (v/v) ethanol treatment for 1 minute. Seeds of *A. laxiflora* were then treated with 1% (w/v) NaOCl solution for 8 minutes and those of *O. apifera* were treated in 0.5% (w/v) NaOCl for 10 minutes.

Sterilisation times and NaOCl solution strength were based on recommendations by Malmgren & Nyström (2014) and observation of seed bleaching from previous studies (Hughes, unpublished data). Petri dishes were subsequently sealed using Parafilm M®. Twenty-five replicate plates of *A. laxiflora* were sown for each of the two treatments giving a total of 50 *A. laxiflora* plates. Due to limited amounts of seed for *O. apifera*, only 15 replicates were sown for each of the two treatments giving a total of 30 *O. apifera* plates. Seed number varied per plate as the sterilisation method did not allow for strict assignment of an exact number of seeds. For *A. laxiflora*, average seed number per plate was 61 (SE = 8) and for *O. apifera*, average seed number per plate was 119 (SE = 17). Fungi were inoculated onto respective symbiotic plates (B1 for *A. laxiflora*, FOA8 for *O. apifera*) via the transfer of a 1 mm^3 piece of fungal culture containing fungal hyphae to the centre of each dish using sterile forceps. Upon completion of fungal inoculation for each orchid species, all instruments were sterilised in 96% (v/v) ethanol before transfer to a glass bead steriliser (Keller Steri 350, Simon Keller AG, Burgdorf, Switzerland) at 250 ºC for 30 seconds. The surface of the laminar flow cabinet was wiped and sterilised with 70% (v/v) ethanol between sessions of working with each different fungi to prevent cross-contamination.

All seed cultures were stored in a dark temperature controlled cabinet at 21±1 ºC, as light has been shown to inhibit germination of *Ophrys sphegodes* Mill. (Mead & Bulard, 1975) and can result in death of protocorms (Mitchell, 1989). Plates were observed for germination and development using a stereomicroscope (Leica M275, Leica Microsystems, Milton Keynes, UK) once every week for 8 weeks. When protocorms had developed to a stage with elongated shoots, cultures were moved into a growth cabinet (Percival AR 66L, Percival Scientific, Perry, IA, USA) and maintained at a diurnal temperature cycle of 17:15 ºC (±1 ºC) and a photoperiod of 14/10 h light/dark under Philips Universal T8 cool white fluorescent lights (Philips Electronics UK, Guildford, Surrey, UK) at 35 µmol m^{-2} s^{-1}. Germination and development was recorded at 5 weeks, 3, 18, and 30 months from the date of sowing. Assessment at 5 weeks and 3 months was done by counting the number of seeds present with and without a visible embryo and noting the number and development stage of protocorms on each plate. Protocorm development was scored using the method proposed by Clements *et al*. (1986): stage 1. germination of seed with rupture of testa; stage

2. production of rhizoids; stage 3. production of leaf primordium; stage 4. production of first chlorophyllous leaf tissue; stage 5. production of root initial, for figure see Chapter 3. This allowed for determination of percentage germination and development stage.

Transfer of plantlets was undertaken once the majority of germianants had developed to stage 5 with green leaves reaching over the height of the petri dishes or when arrested growth was evident. Four to six plantlets were then transferred under aseptic conditions to single Magenta™ GA-7 vessels (Magenta Corp., Chicago, USA) with 60 ml of the appropriate media, sealed with Parafilm M® and returned to the same conditions. This culture regime was continued until 15 months of culture at which point the temperature was increased to a consistent 21±1 ºC in an attempt to induce tuber formation and subsequent summer aestivation. After 18 months culture *in vitro*, plantlets and resultant tubers were de-flasked and all remnants of agar media were removed via careful washing in a sieve under running water. Four to five plantlets were potted in 9 cm^3 pots containing a 2 cm drainage layer of sharp quartzite grit 2 – 6 mm in size, commonly refered to as alpine grit. Next, a moistened compost mixture of Perlite, horticultural sand and John Inness No.1 was added at a ratio of 1:1:1. This was top dressed with a 1 cm layer of alpine grit. Pots were placed in 10 cm deep plastic trays with a 2 cm diameter hole drilled at the base of one side for drainage, the spaces between pots were then in-filled with potting compost. Plantlets were watered and covered with plant propagator lids to help maintain a similar humidity to that of the *in vitro* containers. Lids were then gradually removed over a period of 2 weeks to aid acclimation to ambient humidity. The plantlet trays were maintained in a greenhouse with doors and vents fully open until threat of winter frost, at which point doors and vents were closed. Then a greenhouse heater with frost stat setting was employed and air circulation provided by an internal fan. A watering regime based on a Mediterranean climate was imposed with compost being kept consistently moist from mid-September up until mid-April. Watering was reduced in May with compost staying dry from June until September when watering was recommenced. Small amounts of water were added on a bi-weekly to monthly basis during hot summer periods in order to prevent desiccation of tubers. Assessment was carried out at 18 months; upon de-flasking and at 30 months (after 12 months of *ex vitro* culture) by counting the overall number of

viable plantlets in each treatment. Viable plantlets were determined as those that had reached stage 5 or had advanced to stage 6 (production of tubers).

Percentage germination per plate was calculated after 5 weeks and 3 months culture and data analysed to determine if there was a difference between seeds on the symbiotic and asymbiotic media. A non-parametric Kruskal-Wallis rank sum test was used as the data were not normally distributed and there were some tied ranks. The absolute number of protocorms per development stage were analysed for differences between the two propagation methods using Chi square contingency tests where significance values were calculated using a Monte Carlo simulation based on 10000 replicates (Hope 1968, Patefield, 1981). Analysis was not performed on ex-plants as only absolute values for each treatment were obtained. Statistical analyses were performed using the R statistical framework version 3.2.0 (R core team, 2015) and graphics were constructed using the R programme ggplot 2 (Wickham, 2009).

Results

Final treatment replicate numbers were: 22 plates for the *A. laxiflora* asymbiotic treatment; 24 plates for the *A. laxiflora* symbiotic treatment; 14 plates for the *O. apifera* asymbiotic treatment and 15 plates for the *O. apifera* symbiotic treatment. Replicate numbers per treatment were uneven as some plates were removed from the study due to contamination.

Initial germination and development

On the petri dishes, a total of 200 out of 1477 *A. laxiflora* seeds sown germinated on the asymbiotic media (13.5%) and the maximum development stage obtained at 3 months was stage 3 (Figure 1a; Table 1). A total of 497 out of 1311 seeds sown germinated on the symbiotic media (37.9%) and the maximum development stage obtained at 3 months was stage 5 (Figure 1b, shows an advanced stage 3 symbiotic germinant; Table 1). Percent germination of *A. laxiflora* per plate was significantly higher on the symbiotic cultures compared to the asymbiotic cutures at both 5 weeks ($\chi^2 = 15.88$, df = 1, $p = 6.8 \times 10^{-5}$) and 3 months ($\chi^2 = 23.31$, df = 1, $p = 1.4 \times 10^{-6}$; Figure 2a). Overall, development at 3

months in *A. laxiflora* was considerably more advanced in symbiotic culture compared to asymbiotic culture ($\chi^2 = 328.45$, $p = 1.0 \times 10^{-4}$; Figure 2b). Protocorm death was observed on both treatments, but was low (1 out of 497 protocorms for the symbiotic cultures and 5 out of 200 protocorms for the asymbiotic cultures).

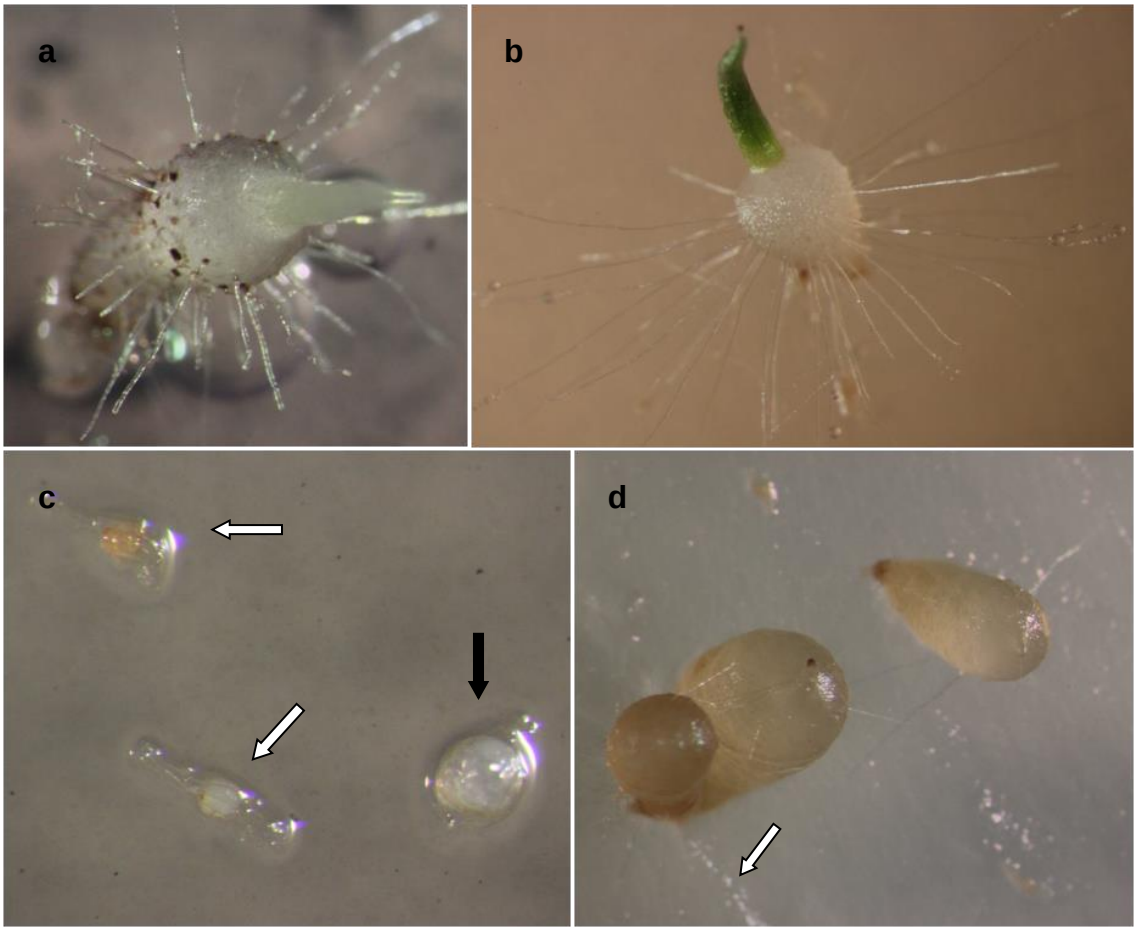

Figure 1. Stages of development at 8 weeks from sowing: **a**) *Anacamptis laxiflora* at stage 3 on Malmgren modified media with potato and pineapple, displaying a leaf primordium (scale bar = 0.5 mm). **b**) *Anacamptis laxiflora* at stage 3 with B1 fungus, showing a leaf primordium differentiating into chlorophyllous leaf tissue (scale bar = 0.5 mm). **c**) Left side (white arrows); ungerminated seed of *Ophrys apifera* on Malmgren modified media with turnip, bottom right (black arrow); germinated seed at stage 1, displaying ruptured testa (scale bar = 0.25 mm). **d**) *Ophrys apifera* at stage 2 with FOA8 fungus, showing formation of rhizoids. Note, the two germinants on the bottom right (white arrow) have germinanted where there is a crack in the media (scale bar = 0.5 mm).

Table 1. Total and median germination percentage results for asymbiotic and symbiotic culture of *Anacamptis laxiflora* and *Ophrys apifera* at 5 weeks and 3 months.

			Total % development stage					Median % development stage			
Anacamptis laxiflora	**Total germination %**	**Median germination %**	**Stage 1**	**Stage 2**	**Stage 3**	**Stage 4**	**Stage 5**	**Stage 1**	**Stage 2**	**Stage 3**	**Stage 4**
5 weeks											
Asymbiotic	9	9	4	5	0	0	0	3	6	0	0
Symbiotic	29	28	0	4	25	0	0	0	4	23	0
3 months											
Asymbiotic	14	13	4	7	3	0	0	2	6	2	0
Symbiotic	38	44	0	4	15	18	1	0	3	10	18

Ophrys apifera	**Total germination %**	**Median germination %**	**Stage 1**	**Stage 2**	**Stage 3**	**Stage 4**	**Stage 5**	**Stage 1**	**Stage 2**	**Stage 3**	**Stage 4**
5 weeks											
Asymbiotic	1	1	1	0	0	0	0	1	0	0	0
Symbiotic	3	0	1	2	0	0	0	0	0	0	0
3 months											
Asymbiotic	2	2	2	0	0	0	0	2	0	0	0
Symbiotic	3	0	0	2	0	0	0	0	0	0	0

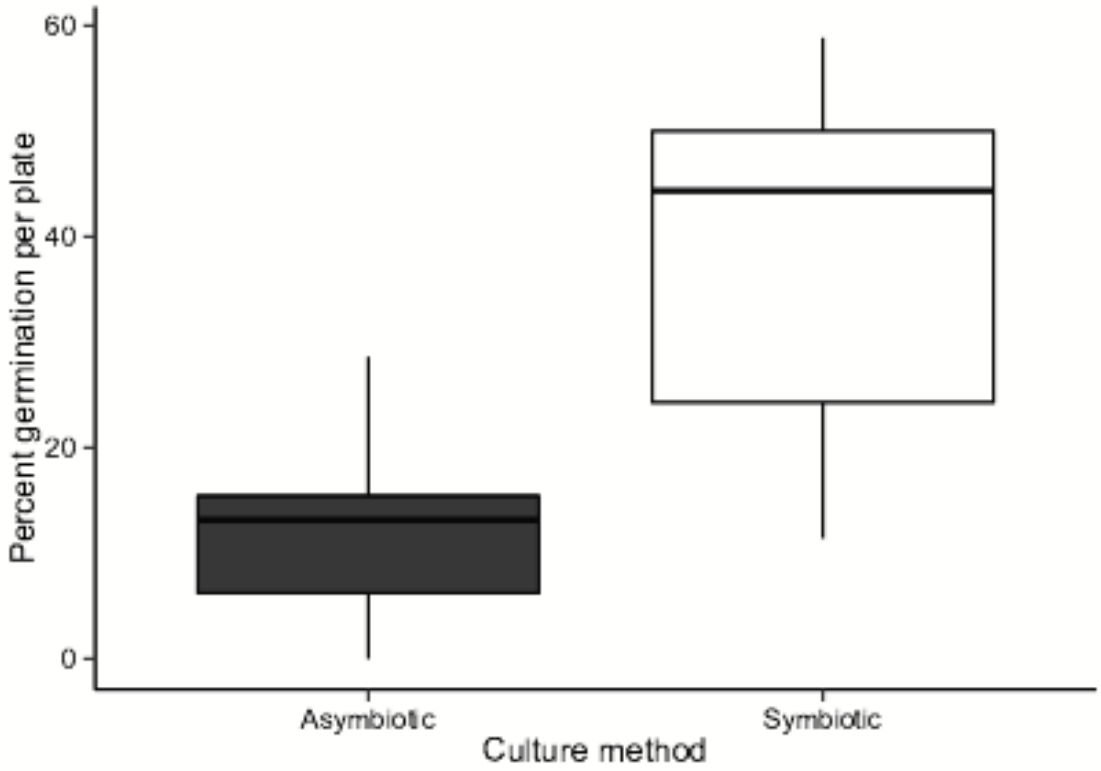

Figure 2. a) Box and whisker plot of percentage germination per plate for *Anacamptis laxiflora* on asymbiotic and symbiotic media at three months from sowing. The line shows the median, the boxes the 25-75% quartiles and the whiskers the spread of the data.

Percent germination of *O. apifera* per plate was far lower than *A. laxiflora*. A total of 33 out of 2008 *O. apifera* seeds sown germinated on the asymbiotic media (1.6%) and the maximum development stage obtained at 3 months was stage 1 (Figure 1c; Table 1). A total of 40 out of 1447 seeds sown germinated on the symbiotic media (2.8%) and the maximum development stage obtained at 3 months was stage 2 (Figure 1d; Table 1). There were no significant differences between percentage germination per plate on the symbiotic and asymbiotic cultures for *O. apifera* at 5 weeks ($\chi^2 = 2.55$, df = 1, p = 0.11), but, at 3 months there was a significant difference between treatments ($\chi^2 = 6.78$, df = 1, p = 0.0092) where germination was higher for the asymbiotic method (Figure 3a). Unlike *A. laxiflora*, symbiotic germination of *O. apifera* was not uniform across plates with many plates exhibiting no germination. There was no germination on nine out of fifteen symbiotic plates compared with no germination on two out of

fourteen asymbiotic plates. Most symbiotic plates where germination was observed had only one protocorm present, however one plate produced 35 protocorms leading to a higher absolute number of germinants with this method. Germination in symbiotic cultures often occurred where there were breaks in the media surface caused by the sowing or fungal inoculating processes (Figure 1d). Overall, development at 3 months in *O. apifera* was significantly more advanced in symbiotic culture compared to asymbiotic culture ($\chi^2 = 47.7$, $p = 1.0 \times 10^{-4}$; Figure 3b). Protocorm death was only observed in the symbiotic treatment (5 out of 40 protocorms).

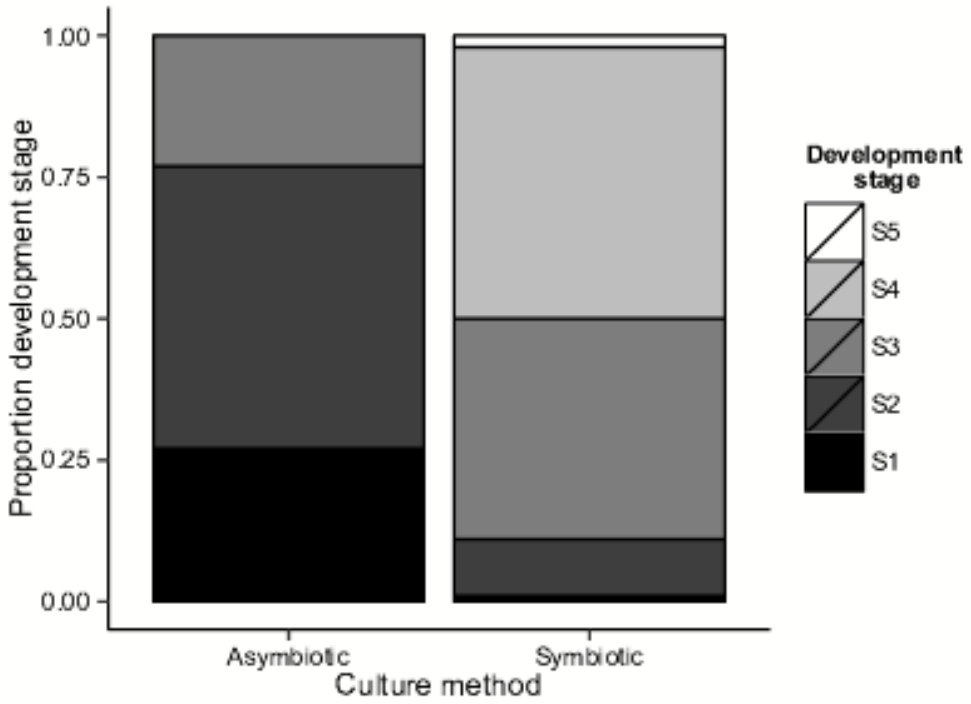

Figure 2. b) Proportion of germinated seed of *Anacamptis laxiflora* at development stages: S1 (Stage 1); S2 (Stage 2); S3 (Stage 3); S4 (Stage 4); S5 (Stage 5) with the asymbiotic and symbiotic method at three months from sowing

Further development and acclimatisation

Development of *O. apifera* largely arrested after 3 months on both treatments with asymbiotic germinants developing only as far as stage 1 and symbiotic germinants as far as stage 2. Transfer of protocorms on both treatments to fresh

respective media did not induce any further development. At 18 months of culture, 111 symbiotic *A. laxiflora* plantlets and 47 asymbiotic plantlets were de-flasked. Unfortunately efforts to induce uniform *in vitro* tuber formation via temperature increase were not effective, with plantlets in random stages of growth at 18 months culture. Some plants were in full growth whilst others were shooting from dormant tubers. After 30 months culture, 12 months of which were *ex vitro*, no asymbiotic ex-plants had survived, however, around 80 symbiotic ex-plants survived with some asexual propagation taking place amongst larger plants (Figure 4). At 36 months culture the first flower bud was observed.

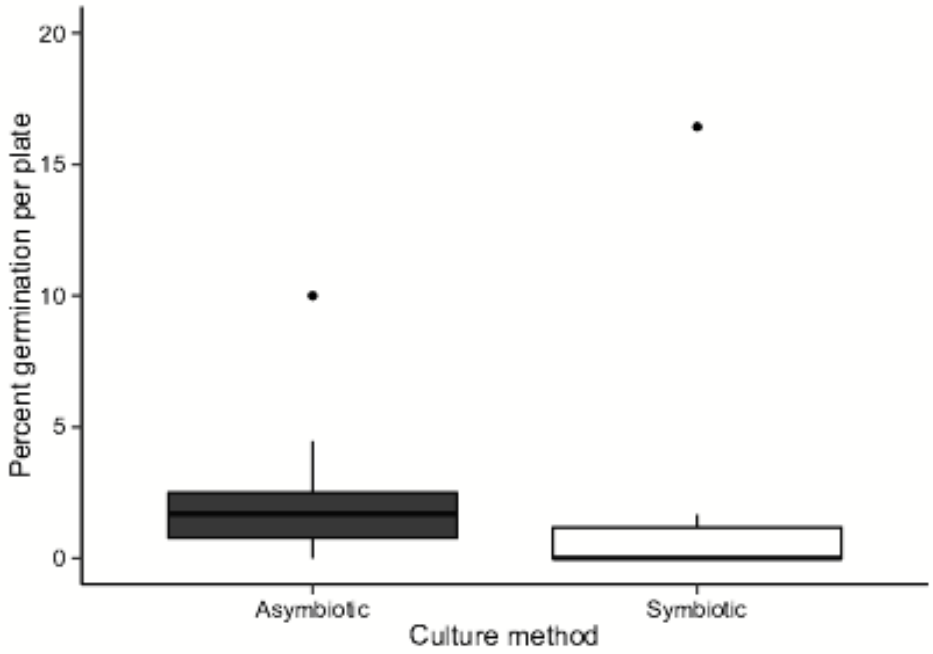

Figure 3. a) Box and whisker plot of percentage germination per plate for *Ophrys apifera* on asymbiotic and symbiotic media at three months from sowing. The line shows the median, the boxes the 25-75% quartiles and the whiskers the spread of the data. Outliers are shown by dots.

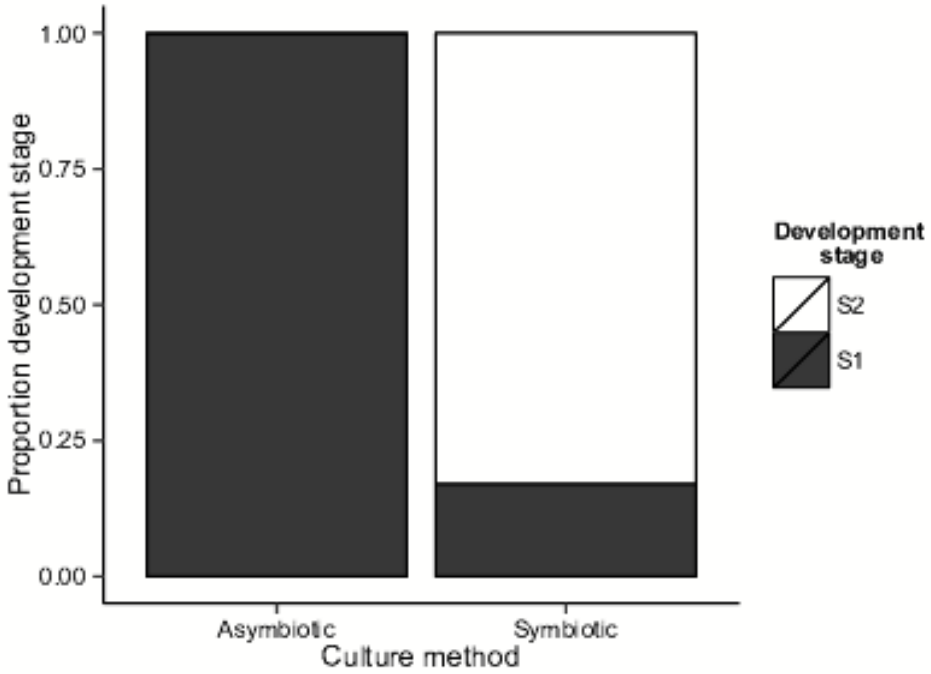

Figure 3. b) Proportion of germinated seed of *Ophrys apifera* at development stages: S1 (Stage 1); S2 (Stage 2) with the asymbiotic and symbiotic method at three months from sowing.

Figure 4. *Anacamptis laxiflora* plants with B1 fungus in cultivation in a temperate greenhouse at 26 months from sowing (8 months culture *ex vitro*).

Discussion

The hypothesis that symbiotic propagation would be most effective in germinating *A. laxiflora* and *O. apifera* proved to be generally correct in this study. Germination of *A. laxiflora* with the symbiotic method resulted in around three times more germinants than the asymbiotic method. Germination in *O. apifera* however, was very low on both treatments when compared with *A. laxiflora*. The symbiotic method proved most effective in germinating *O. apifera* overall, with almost twice the relative number of germinants obtained compared with the asymbiotic method. However, variability was greater across plates and *O. apifera* germinated more consistently on the asymbiotic media. After 3 months culture on both treatments, growth of *O. apifera* germinants had arrested with asymbiotic germinants developing only as far as stage 1 and symbiotic germinants as far as stage 2. Efforts to induce further development by transfer of protocorms to fresh respective media were not successful. At 3 months from sowing symbiotic plantlets of *A. laxiflora* were considerably more advanced than asymbiotic plantlets. By 18 months culture, more than double the number of symbiotic plantlets compared with asymbiotic plantlets were suitable for planting *ex vitro*. After a further 12 months culture *ex vitro*, no asymbiotic plantlets had survived compared to around 80 symbiotic ex-plants surviving with flowering observed 6 months later.

Although extensive literature exists separately on the asymbiotic and symbiotic propagation of orchids, little work has been done comparing the two methods. Most notable of the comparative studies are the work of Muir (1989), Rasmussen *et al*., (1990a), Anderson (1991) and Johnson *et al.,* (2007), which focused on terrestrial orchids, while Otero & Bayman (2009) undertook research on epiphytic orchids. These studies showed variable results. Muir (1989) found, no significant differences in germination for three *Anacamptis* species, including *A. laxiflora*. Rasmussen *et al*., (1990a) however, found higher germination and faster development in symbiotic cultures of *Dactylorhiza majalis* (Rchb.) when compared with asymbiotic cultures. Anderson (1991) also obtained similar results with *Spiranthes magnicamporum* Sheviak. Conversely, for the terrestrial species *Eulophia alta* (L.) Fawc. & Rendle, Johnson *et al.* (2007) found higher germination with the asymbiotic method. Otero & Bayman (2009) found significantly higher germination and more advanced development for the

epiphytic *Tolumnia variegata* (Sw.) Braem, with symbiotic fungi isolated from the same orchid species. When the same fungi were combined with other more distantly related epiphytic orchids there were no significant differences between both methods (Otero & Bayman, 2009). In all cases, the tested symbiotic fungi were isolated from either the focal orchid species, an orchid species within the same genus or from other orchids occupying similar habitats. This should maximise the possibility that they would be suitable symbionts. However, differences in fungal species, specific media used, and the growing conditions employed could all contribute to the varying levels of success observed between treatments. While the current study does not directly address this issue, it does provide additional evidence supporting the relative success of symbiotic methods.

Symbiotic germination of *O. apifera* in my study was not uniform, with many plates exhibiting no or very little germination. These results are somewhat consistent with the observations of Ted Weeks (personal communication, 2013), who reported sporadic germination, with certain plates containing high numbers of germinants and others none. A similar conclusion was reached by Muir (1989) with *O. sphegodes*. In this experiment, seed germination in symbiotic cultures of *O. apifera* often occurred where there were breaks in the media surface caused by the sowing or fungal inoculating process. Thus, changes in the rugosity of the media may have affected the growth of the fungus and its ability to germinate *O. apifera*. Considering that a horizontal agar plate is quite different to the three dimensional structure of the soil and plant root environment that the fungus would naturally colonise, it may be that the morphology of the fungus grown on flat media is not identical to its native state. Further germination studies utilising sterile soil or compost-based media inoculated with a symbiotic fungus (see Quay *et al*., 1995 for an example with Australian terrestrial orchids) could provide interesting results.

Germination for *O. apifera* in this study was particularly low on both media. Levels of germination in previous studies on *Ophrys spp.* tend to be highly variable with some experiencing low (Veyret, 1969) and others high (Borris & Albrecht, 1969; Malmgren, 1989; Malmgren & Nyström, 2014, Ponert *et al.*, 2011) germination rates. In a comparison of asymbiotic propagation techniques of terrestrial orchids, Ponert *et al.* (2011) concluded that there was no link between

the type of media used, the sterilisation times or techniques used and germination rates achieved with this genus. Such variability in results among studies may, to some extent, be due to differences in seed viability or a maternal effect due to parent plant conditions that is inherent in the seeds. The most successful germination of *O. apifera* in the literature was achieved by Kitsaki *et al*. (2004) with a germination rate of 95% using immature seed. The seed of *O. apifera* used in this study was mature and 3 years old at the time of sowing and had likely lost viability over time. Unfortunately insufficient seed was available at that time to test viability *a priori*.

The hypothesis that symbiotic fungi would promote development proved to be correct for both orchid species tested. Development in *A. laxiflora* was far more advanced in symbiotic culture with the majority of germinated seeds beginning to form chlorophyllous leaf tissue and a small proportion resembling miniature plantlets after only three months. By comparison, development of germinants in asymbiotic culture over the same time period was retarded, with the majority remaining at the protocorm stage with rhizoids. Although germinants of *O. apifera* were not as advanced in their development as *A. laxiflora*, they also developed furthest with the symbiotic method. On the symbiotic media, most protocorms developed rhizoids whereas on the asymbiotic media, the germinants only had ruptured testas after the same time point. As in this study, Muir (1989) observed the most advanced development when utilising symbiotic fungi, with many germinants developing to form green leaves compared with the maximum development of shoot primordia on asymbiotic media after five weeks of culture. A relevant point discussed by Muir (1989) and also highlighted by Malmgren & Nyström (2014) is that many orchids develop only to a certain stage on some asymbiotic media, which could explain the slower development seen in my study. Re-plating to fresh media or to one with a different formulation, for example one containing a higher sucrose content or different complex organic ingredients, can often solve this problem (Rasmussen, 2006; Pierce *et al.*, 2015). Different asymbiotic media formulations were not tested in this study, but instead we demonstrated that *A. laxiflora* plantlets produced on the symbiotic media were a significantly more advanced at 3 months from sowing. At 18 months from sowing, the symbiotic method had produced more than double the number of plantlets suitable for planting *ex vitro* than the asymbiotic method and after a further 12

months *ex vitro* culture no asymbiotic plantlets had survived. These results similarly reflect those observed by Anderson (1991) and Aggarwal & Zettler (2010). Symbiotic methods, therefore, can potentially reduce the time of *in vitro* culture, making for a cheaper and more fruitful propagation with higher survival rates *ex vitro*, and may prove especially useful where asymbiotic plantlet establishment has failed.

Compared to *A. laxiflora*, the development observed in *O. apifera* was less advanced and only produced protocorms with rhizoids. Previously, Muir (1989) showed that although *Ophrys spp*. germinated symbiotically and developed to form early stage protocorms on a variety of fungal isolates, further development to form plantlets only occurred on fungal isolates obtained from the *Ophrys* genus. The fungal isolate used in this study for *O. apifera* originated from protocorms of the same species. It is possible that this isolate also only supports initial germination and therefore, additional fungi may be required to induce further development of this species. However, Ted Weeks (personal communication, 2013) reported successful germination and plantlet development of *O. apifera* and its varieties using this fungus. Unlike *O. apifera*, *A. laxiflora* appeared to be less exacting in its choice of fungal partner. The fungal isolate used here with *A. laxiflora* was B1, which was isolated from *D. fuchsii*. The B1 isolate belongs to the *Ceratobasidium* group of fungi (Heys, 2012), which have been shown to associate with and germinate a number of orchids (Hadley, 1970; Warcup, 1973, 1981; Clements *et al.*, 1986, Bonnardeaux *et al*., 2007; Girlanda *et al*., 2011). The results of this study further supports the usefulness of the B1 isolate, which has been widely used by hobbyists to successfully germinate a number of terrestrial species (Alan Leck, personal communication, 2012).

Although asymbiotic propagation methods are able to produce large numbers of sterile orchid plantlets, my results and those of Muir (1989); Rasmussen *et al*., (1990a); Anderson (1991); Otero & Bayman (2009) demonstrate that symbiotic propagation can offer faster development with appropriate fungi. This study and those of Anderson (1991) and Aggarwal & Zettler (2010) have shown that symbiotic methods can also offer higher rates of survival *ex vitro*. The increased symbiotic seedling survival and rates of development are likely due to the benefits of mycorrhizal association. These potentially protect seedlings from pathogenic microorganisms and provide a

mycotrophic capability, allowing the plants to digest fungi as an additional energy source (Rasmussen, 2006). In the case of this experiment, the loss of asymbiotic plantlets *ex vitro* may be explained by their comparatively retarded development when compared to symbiotic seedlings at the same time point. This may have resulted in the formation of fewer and smaller tubers in *ex vitro* plantlets, which may have had a negative impact on aestivation survival (Batty *et al*., 2006a). Additionally, the inconsistencies in tuber formation after the imposed summer temperature increase may have resulted in the losses of plants in both treatments. This failure may be due to the need for higher summer temperature than that provided, a result of the higher moisture experienced in *in vitro* culture or other abioitic or biotic cues. In order that *in vitro* orchid germination and development is in sync with that of its native habitat, Malmgren & Nyström (2014) recommend following the natural sowing and associated temperature cycle that orchid seeds and germinants would experience in their natural environment. Investigating whether implementing a more detailed sowing and temperature cycle would prove useful in a future study.

Symbiotic methods may also prove more effective in propagating difficult genera, especially those with a high reliance on mycorrhizal fungi (Umata 1995, 1998a, 1998b, Yagame *et al*. 2007) and have already been shown to be beneficial in conservation projects. For example; Stewart *et al*., (2003) utilised symbiotic techniques to successfully reintroduce the rare terrestrial orchid *Spiranthes brevilabris* Lindl. and Zettler *et al*., (2007) the epiphytic orchid *Epidendrum nocturnum* Jacquin into suitable natural habitats. Aggarwal & Zettler (2010) also had similar success with reintroduction using symbiotically grown seedlings where previous attempts using asymbiotic seedlings had failed. Muir (1989); Anderson, (1991) and Wood & Ramsay (2004) also reported similar success introducing symbiotically grown plants into a semi-natural setting. Finally, in addition to facilitating future recruitment and establishment of orchids, the use of symbiotic methods for reintroductions could also be used to introduce appropriate communities of mycorrhizal fungi into habitats with degraded soils, thus helping to restore their former function (Zettler, 1997a). In conclusion, the results of this study add further credence to the utility and advantage of symbiotic techniques for the propagation of orchid species, whether for conservation or horticulture.

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Chapter 5.

The effects of substrate rugosity and complexity on *in vitro* germination and development in the European terrestrial orchids; *Anacamptis morio* and *Dactylorhiza purpurella*

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Abstract

Terrestrial orchids are highly reliant on mycorrhizal fungi for successful germination and development. Determining effective methods to propagate terrestrial orchids could assist the production of viable plants for horticulture and conservation efforts. It was hypothesised that increased media rugosity would have a positive effect on symbiotic terrestrial orchid germination and development *in vitro*. To ascertain this, seeds of two European terrestrial orchids; *Anacamptis morio* (L.) R. M. Bateman, Pridgeon & M. W. Chase and *Dactylorhiza purpurella* (T.Stephenson & T.A.Stephenson) Soó were sown on agar based media with either flat or cut treatments. To determine whether substrate complexity had an effect; inorganic and soil based media were tested. These media treatments were split into asymbitoic and symbiotic treatments using either asymbiotic Malmgren modified media or symbiotic H1 media with the B1 fungal isolate (*Ceratobasidium sp*. Anastamosis Group C). Germination and development were recorded at 10 and 20 weeks from sowing.

It was demonstrated that increased substrate rugosity on agar based media had a positive effect on symbiotic germination in *A. morio* on symbiotic cultures, and with *D. purpurella* in both symbiotic and asymbiotic cultures. Increased development in these cultures also appeared to be supported. The effect of the method in agar based cultures was also highly significant in *D. purpurella* with higher germination in symbiotic compared to asymbiotic treatments. The magnitude of the rugosity effect in both orchid species was dependent on whether a symbiont was present. *Anacamptis morio* showed a significant response to substrate complexity upon germination in symbiotic cultures across all treatments whereas *D. purpurella* showed a significant effect of substrate complexity across treatments with the asymbiotic and symbiotic method. These results show that substrate rugosity and complexity are important factors in the germination of these terrestrial orchids *in vitro*, with implications for orchid-fungal studies and propagation.

Keywords Orchidaceae • Germination • Terrestrial • Mycorrhizae • Rugosity • Substrate complexity

Introduction

The family Orchidaceae is one of the most widely distributed and speciose of all plant families with the latest count at 28,484 (WCSP, 2017), around a third of which are terrestrial orchids (Swarts & Dixon, 2009a). Orchids have evolved a number of specialisms such as specific insect pollinator interactions and a reliance on specific fungi for germination and development. Such specialisms often result in many species being restricted to niche habitats and narrow ranges. This puts them at particularly high risk of human impacts, which are actively causing habitat loss and degradation, due to overexploitation and changes in climate (Cribb *et al*., 2003; Swarts & Dixon, 2009a). Terrestrial orchids are therefore considered to be some of the most vulnerable of the higher plant groups (Zettler, 1997b) with a large proportion considered to be rare, threatened or endangered (Whigham & Willems, 2003), and therefore require active conservation *in situ* and where necessary *ex situ*. Studying propagation methods *ex situ* is vital for many rare species where populations have become unviable due to severe habitat reduction and fragmentation (Swarts & Dixon, 2009a). Gaining a better understanding of orchid-mycorrhizal associations could help improve methods to propagate and conserve these orchids.

Ex situ propagation of terrestrial orchids is most often achieved using either asymbiotic or symbiotic methods. Both methods commonly utilise agar based media. In the case of the asymbiotic method, seeds are sown without fungi on a sterile nutrient media which can contain a variety of nutrients, sugars, trace elements, amino acids, vitamins, phytochemicals and other complex organic substances. These essentially aim to replace those that the fungi may provide during symbiosis (Rasmussen, 2006; Seaton *et al*. 2011). Symbiotic methods in contrast, utilise a mycorrhizal fungus which is cultured in association with the orchid seed. The media for symbiotic culture differs from asymbiotic media by the inclusion of polysaccarhides; cellulose (Hadley, 1969) and starch in the form of oatmeal (Clements & Ellyard, 1979; Clements, 1982a; Rasmussen, 2006). These carbohydrates are not accessible directly to orchid germinants, but are instead broken down into more accessible forms such as monosaccharides via the fungal metabolism (Rasmussen, 2006).

A variety of fungi have been used successfully in symbiotic germination studies. They are primarily basidiomycetes and are often split into sexual teleomorphic and asexual anamorphic genera (in parenthesis). These include: *Tullasnella (Epulorhiza)* (Hadley, 1970; Warcup, 1971; 1973; 1975; 1981; Stewart *et al*., 2003; Rasmussen, 2006); *Serendipita* (Warcup, 1971; 1981; 1988), *Ceratobasidium (Ceratorhiza)* Hadley, 1970; Warcup, 1973; 1975; 1981; Muir, 1989; Rasmussen, 2006; Aggarwal & Zettler, 2010) *Thanatephorus (Rhizoctonia)* (Hadley, 1970; Warcup, 1975; 1981; Clements *et al*., 1986; Masuhara & Katsuya, 1994; Rasmussen, 2006). Successful associations are dependent on the orchid species, many of which will have differing fungal specificities during germination and development (Warcup, 1981, 1985; Kulikov & Filippov, 2001; Rasmussen, 2006; Bidartondo & Read, 2008). Fungal compatibility trials as in Chapter 2, 3 can prove a useful way to determine optimal strains for terrestrial orchid propagation.

The results observed in Chapters 2; 3 and 4, have shown varied and sometimes unusual germination and developmental distributions of terrestrial orchids across symbiotic treatments on agar based media *in vitro*. Orchid seeds can often prove very difficult to germinate, with different investigators reporting varying results that can prove difficult to replicate (Hughes, unpublished data; Rasmussen, 2006; Ponert *et al*., 2011). There are many abiotic and biotic factors potentially affecting seed germination: seed and embryo variability; seed sterilisation and scarification; cold stratification; temperature; light; moisture level; nutrient distribution and availability; fungal hyphal density and the compatibility of the fungal strain (Arditti & Ghani, 2000; Rasmussen, 2006). As a result of the observations made in Chapter 4, it was postulated that there may have been an effect of substrate rugosity and complexity on the germination and development in symbiotic cultures. This study was designed to investigate this effect.

Here, two species; *Anacamptis morio* (L.) R.M.Bateman, Pridgeon & M.W.Chase, the Green–winged Orchid and *Dactylorhiza purpurella* (T.Stephenson & T.A.Stephenson) Soó, the Northern Marsh Orchid, from the orchid tribe Orchideae were chosen based on availability of seed as well as previous germination success using asymbiotic and symbiotic methods (Hughes, unpublished data, Chapter 3.) A third species, *Ophrys apifera* Huds. (see, Chapter 4) was not included due too poor germination across treatments. Both

included species have potential horticultural value and can potentially be used as models for other species within their genera. In addition, *Anacamptis morio* is listed as near threatened on the Red List of Vascular Plants for Great Britain (Cheffings *et al*., 2005) and Europe (Bilz *et al*., 2011) and may benefit from *ex situ* propagation followed by reintroduction efforts. Many species within these genera including *A. morio* are subject to heavy collection pressures from the Salep trade (Sazak & Ozdener, 2006; Ghorbani *et al*., 2014; Kemeç *et al*., 2015).

The aim of this study was to test the hypothesis that increased substrate rugosity would have a positive effect on the symbiotic germination and development in the European tuberous terrestrial orchids; *A. morio* and *D. purpurella*. The objectives were to ascertain if substrate rugoisty and different levels of substrate complexity affected symbiotic and asymbiotic germination and development in these orchid species. Developing a greater understanding of orchid-fungal symbiosis could help to refine conservation efforts and develop more effective propagation methods.

Materials and methods

Organismal details

Anacamptis morio, the Green–winged Orchid is a tuberous geophyte, producing a wintergreen rosette and aestivating as globose rootless tubers. It is Euro-Mediterranean in distribution, growing as far north as Britain and southern Norway and east to Iran. It can be found up to 2000 m in a number of habitats in full sun such as short, poor grassland, alpine pastures, unimproved meadows, as well as open woodland and forest fringes in the south of its range (Delforge, 2006; Rasmussen, 2006; Harrap & Harrap, 2009). *Dactylorhiza purpurella*, the Northern Marsh Orchid is a tuberous geophyte, producing summergreen leafy shoots, which originate from palmately divided tubers. It has a more limited distribution to Atlantic northwestern Europe and is found in Britain, Ireland and Scandinavia. It can be found up to 500 m on calcareous, neutral to slightly acidic, moist and wet soils in full sun in a variety of damp habitats such as marshy fields, damp meadows, fens, marshes, dune slacks, damp coastal meadows and occasionally

drier dune slopes and open damp woodland. (Delforge, 2006; Rasmussen, 2006; Harrap & Harrap, 2009).

The symbiotic fungus B1 was obtained from the Hardy Orchid Society Seed Bank (UK). This fungus was isolated from the root of *Dactylorhiza fuchsii* (Druce) Soó, collected in Avon, UK by a member of the Hardy Orchid Society (Jim Hill). This isolate was chosen because it has been shown to be effective at germinating and producing viable plantlets of *Anacamptis* and *Dactylorhiza ssp.* (Alan Leck, personal communication, 2012; Hughes, Chapter 3). DNA analysis in 2011 established it as a type of *Ceratobasidium sp*. from Anastamosis Group C (Heys, 2012).

Experimental procedure

Mature, open pollinated seed pods of *Anacamptis morio* were collected in September 2015 from the Eastoft Meadow Site of Special Scientific Interest (SSSI), a lowland mesotrophic 'neutral' grassland habitat designated as old grazed hay meadow; MG5 *Centaurea nigra – Cynosurus cristatus* (Rodwell, 1998) in North Lincolnshire, England, UK with permissions from the land owner and Natural England. Mature, open pollinated seed pods of *Dactylorhiza purpurella* were collected in September 2015 from plants growing in the grounds of the University of Manchester, Manchester, UK. For each species, seeds from different individuals and seed pods were combined, placed in greaseproof paper packets and dried in a plastic seed drying box with silica gel for 2 weeks at room temperature. Seed packets were then stored in an air-tight container with silica gel at 3±1 ºC until use. The B1 fungus was sub-cultured on Potato Dextrose Agar, (Formedium, Hunstanton, UK) under aseptic conditions in a laminar flow cabinet (Flowfast V 15P, Faster D:Group, Ferrara, Italy) and stored in a dark temperature controlled cabinet (LMS Series 4 Model 1200, LMS Ltd., Sevenoaks, Kent, UK) at 21±1 ºC prior to use as fungal inoculant.

The following factors among treatments and within species were standardised: seed sterilisation technique; media volume; vessel size; temperature regime, light level and photoperiod. Seeds of both species were sown in August 2016 on 90 mm sterile petri dishes (Sterilin™, Thermo Fisher Scientific, Newport, UK) containing 30 ml media and sealed with Parafilm M® (Bemis, Neenah, WI, USA). Seeds were sown on a number of different sterile

media with differing rugosity and complexities. To test rugosity, an agar based media with either flat or cut treatments was used. In the case of the cut treatment, cuts were made at 1 cm intervals in a cross-hatch pattern using a scalpel. Therefore, this allowed for the cut treatment to be divided into cut and uncut sections for further analysis. The presence of cuts allowed for seeds to fall either on cut or uncut sections of the plates during sowing. These within plate differences were used to test for rugosity, and to compare seeds growing on the uncut sections to seeds growing on flat treatments (i.e. uncut media) as a control. To test for substrate complexity, an inorganic media of vermiculite and horticultural sand (V/S) at the ratio of 1:1 and a soil based media using John Innes (JI) No. 1 were used. These media treatments were split into asymbiotic and symbiotic treatments using either asymbiotic Malmgren modified media (M+PP) (Malmgren 1996; Malmgren & Nyström, 2014) or symbiotic H1 media (Clements *et al*., 1986, modified by Rasmussen *et al*., 1990a) with the B1 fungal isolate (H1+B1). Malmgrem modified media has been used widely in the asymbiotic propagation of terrestrial orchids (Kitsaki *et al.,* 2004; Malmgren, 1996; Pierce & Cerabolini, 2011; Pierce *et al.*, 2010, 2015, Hughes, Chapter 3, 4). H1 Oat Medium has been extensively used for the symbiotic culture of terrestrial orchids (Clements *et al*., 1986; Muir, 1989; Rasmussen *et al*., 1990a, 1990b; Wood & Ramsay, 2004, Hughes, Chapter 2, 3, 4). In the case of vermiculite/horticultural sand and John Innes No. 1 treatments, 6 ml of the same asymbiotic and symbiotic media formulations with the omission of agar were dispersed evenly over the media surface on each plate.

Seed sterilisation was conducted under aseptic conditions in a laminar flow cabinet using the sterile syringe and mesh method (Ponert *et al*. 2011) with 48 micron nylon mesh (Plastok, Birkenhead, UK) and an initial 70% (v/v) ethanol treatment for 1 minute. Seeds of *A. morio* were then treated with 1% (w/v) NaOCl solution for 8 minutes and those of *D. purpurella* were treated in 1% (w/v) NaOCl for 20 minutes. Sterilisation times and NaOCl solution strength were based on recommendations by Malmgren & Nyström (2014) and observation of seed bleaching from previous studies (Hughes, unpublished data). Petri dishes were subsequently sealed using Parafilm M®. Fifteen replicate plates were sown for each treatment giving a total of 120 *A. morio* and 120 *D. purpurella* plates. Seed number per plate varied as the sterilisation method did not allow for exact

numbers of seed to be allocated to each plate. For *A. morio*, average seed number per plate was 11.66 (SE = 1.06) and for *D. purpurella*, average seed number per plate was 61.32 (SE = 5.60). The fungal isolate was inoculated onto symbiotic plates via the transfer of a 1 mm^3 piece of fungal culture containing fungal hyphae to the centre of each dish using sterile forceps. Upon completion of fungal inoculation for each orchid species, all instruments were sterilised in 96% (v/v) ethanol before transfer to a glass bead steriliser (Keller Steri 350, Simon Keller AG, Burgdorf, Switzerland) at 250 ºC for 30 seconds. The surface of the laminar flow cabinet was wiped and sterilised with 70% (v/v) ethanol between sessions of working to prevent contamination.

All seed cultures were stored in a dark temperature controlled cabinet at 21±1 ºC. Germination and development on petri-dishes was observed using a stereomicroscope (Leica M275, Leica Microsystems, Milton Keynes, UK) once every week for 10 weeks. Once protocorms had developed elongated shoots, cultures were moved to a growth cabinet (Percival AR 66L, Percival Scientific, Perry, IA, USA) and maintained at a diurnal temperature cycle of 17:15 ºC (±1 ºC) and a photoperiod of 14/10 h light/dark under Philips Universal T8 cool white fluorescent lights (Philips Electronics UK, Guildford, Surrey, UK) at 35 µmol m^{-2} s^{-1}. Germination and development was recorded at 10 weeks and 20 weeks from the date of sowing. This was assessed by counting the number of seeds present with and without a visible embryo and noting the number and development stage of protocorms on each plate. Protocorm development was scored using the method proposed by Clements *et al*. (1986): stage 1. germination of seed with rupture of testa; stage 2. production of rhizoids; stage 3. production of leaf primordium; stage 4. production of first chlorophyllous leaf tissue; stage 5. production of root initial, for figure see Chapter 3. This allowed for determination of mean percentage germination and development stage. For each treatment, total numbers of germinants at specific development stages and overall germination were recorded for each plate. From this, percentages of germinants at each development stage and germination totals were calculated for each plate and means taken for each treatment along with the standard error of the means. Statistical analysis was performed with germination data at 20 weeks of culture, using 3 analyses. Data was not analysed from week 10 due to minimal changes in germination between weeks 10 and 20.

Analysis 1. Cut vs uncut sections of agar based media, inside the same plate; rugosity treatment.

The proportion of germinated seeds was calculated for both orchid species from each plate of the cut treatments on both symbiotic and asymbiotic media. Seeds or germinants were categorised as 'cut' if they were found within or emerging from a cut in the media. Seeds or germinants were categorised as 'uncut' if they were located on a flat, uncut area of the media. For each species, to determine whether there were effects of the rugosity treatment, culture method (asymbiotic vs symbiotic), or the interaction between them on germination, observed differences were compared in the proportion of germinated seeds to a null distribution of 10^5 randomly generated permutations of the data. Each plate was treated as a data point, and to create permutations, data points were reassigned between treatments. The p-value is the proportion of the null distribution in which the difference between treatments was as great as, or greater than, the observed difference.

Analysis 2. Negative control; uncut sections of cut treatments vs flat (uncut) treatments with agar based media

The proportion of germinated seeds was calculated for each orchid species on each culture method (symbiotic and asymbiotic), in the uncut sections of the cut treatment plates and the entire non-treated flat treatment plates. The aim of this analysis was to determine whether there were effects of the cut treatment on the germination of seeds in the uncut sections of the plate. To do this, the differences in the proportion of germinated seeds in the uncut sections and the non-treated, flat plates was calculated for each species and method. As above, a resampling method was used to test for significance.

Analysis 3. Comparison across multiple substrates for each species

To analyse the effect of substrate complexity on the proportion of germinates, a PERMANOVA analysis with 1000 permutations was performed. This method allowed us to test the effect of substrate complexity even if the data is not normally distributed. A post-hoc analysis was obtained by performing pairwise comparisons between the different substrates and applying Bonferroni correction for multiple comparisons. These analyses were performed with the package

"vegan" (Oksanen *et al*., 2017) in the R statistical environment (R core team, 2017).

Results

Final treatment replicate numbers for *A. morio* were: 11 plates for asymbiotic M+PP flat treatment; 12 for symbiotic H1+B1 cut; 13 plates for asymbiotic M+PP cut treatment and 15 plates for each of; asymbiotic JI M+PP, V/S M+PP, symbiotic H1+B1 flat, H1+B1 cut, JI H1+B1 and V/S H1+B1. For *D. purpurea* there were 13 plates for asymbiotic M+PP flat treatment and 15 for each of; asymbiotic M+PP cut, JI M+PP, V/S M+PP, symbiotic H1+B1 flat, JI H1+B1 and V/S H1+B1. Replicate numbers per treatment were uneven due to the loss of some plates due to contamination or lack of seed.

Germination and development

Germination in *D. purpurea* at week 10 was highest on the H1+B1 cut treatment (42.8%±3.6 with 54.5%±5.4 on cut and 36.3%±3.7 on uncut sections within treatment), followed by H1+B1 flat (34.8%±3); V/S H1+B1 (31.3%±6.5); M+PP cut (15.6%±1 with 17.3%±2.4 on cut and 15%±1.1 on uncut sections within treatment); M+PP flat (13.3%±3.1SD); JI H1+B1 (13%±4.2); JI M+PP (7%±1.4) and V/S M+PP (1.5%±0.6; see Table 1). At week 20 the same trend continued with overall increases in germination observed on treatments; H1+B1 cut (43.3%±3.6 with 54.7%±5.5 on cut and 36.9%±3.6 on uncut sections within treatment); H1+B1 flat (35.8%±2.9); V/S H1+B1 (32.1%±6.5); M+PP cut (17.2%±1.3 with 25%±3 on cut and 14.8%±1 on uncut sections within treatment) whereas treatments; JI H1+B1; JI M+PP and V/S M+PP did not show any further increase in germination. Treatment M+PP flat (13.1%±3.1) in contrast had a decrease in overall viable germination due to an increase in necrotic germinants (Table 2; Figure 1). Protocorm death was only observed in *D. purpurea* and only occurred on asymbiotic; flat treatments and uncut sections of cut treatment at development stage 1 and 2, with protocorm death consisting of no more than 2.2% at week 10 and 4.4% at week 20 of overall germination on these treatments.

At week 10 *A. morio* germinated highest on symbiotic JI H1+B1 with a mean percentage germination per plate of 27.1%±3.7, followed by H1+B1 flat

(18.3%±3.8); M+PP cut (14.5%±2.4 with 15.8%±7.6 on cut and 11.8%±4.3 on uncut sections within treatment); V/S H1+B1 (12.6%±5.1); H1+B1 cut (9.5%±3.5, with 27.1%±8.8 on cut and 3.3%±3.3 on uncut sections within treatment); M+PP flat (8.2%±2.7; see Table 1). No germination was observed on either asymbiotic JI M+PP or V/S M+PP. At week 20 the same trend continued, but there was no overall increase in germination except for JI H1+B1 (29.8%±3.4); M+PP cut (16.1%±2.8 with 19.5%±7.9 on cut and 17.4%±7.5 on uncut sections within treatment); V/S H1+B1 (14.3%±6.1), see Table 2; Figure 2.

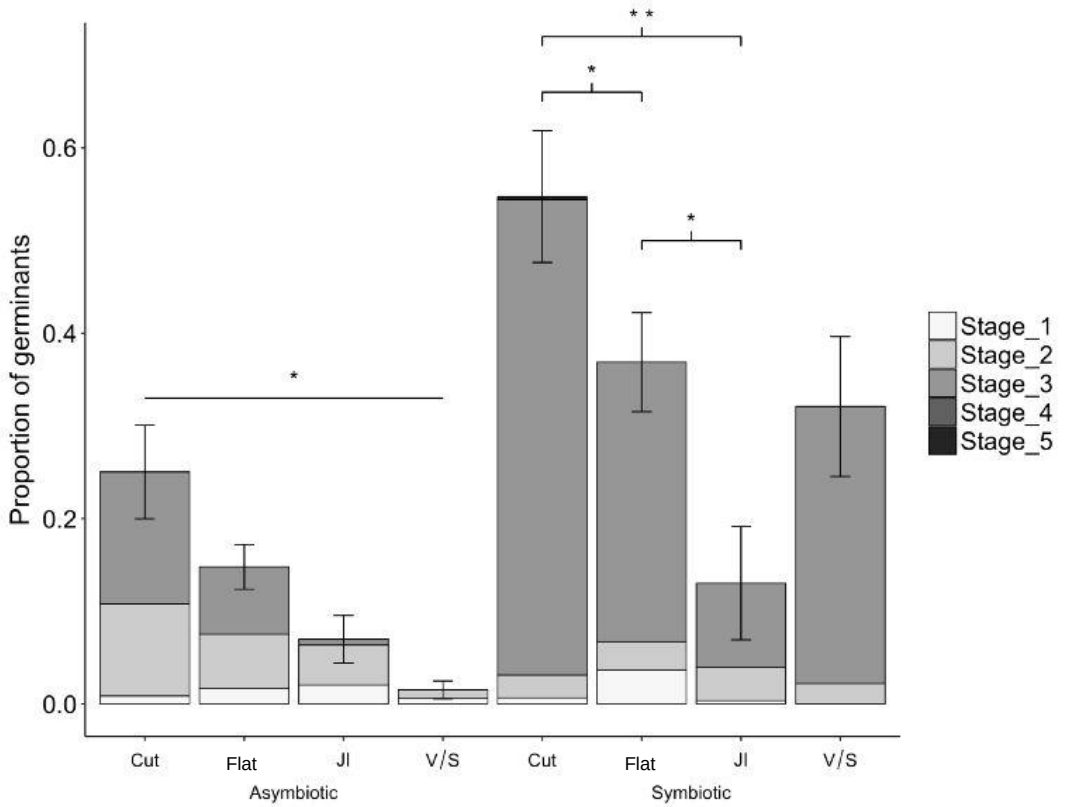

Figure 1. Mean numbers of germinated seed at different development stages for *Dactylorhiza purpurella* on asymbiotic Malmgren or H1 symbiotic media with B1 fungus on the following substrates: Cut (cut sections of cut agar based media; Flat (uncut agar based media); JI (John Innes No.1 + liquid media); V/S (Vermiculite and horticultural sand + liquid media) at 20 weeks from sowing. Development stage 1 (white); stage 2 (light grey); stage 3 (grey); stage 4 (dark grey); stage 5 (black). Error bars represent the standard error. * denote significant difference of <0.05; ** denote significant difference of <0.01; *** denote significant difference of <0.001.

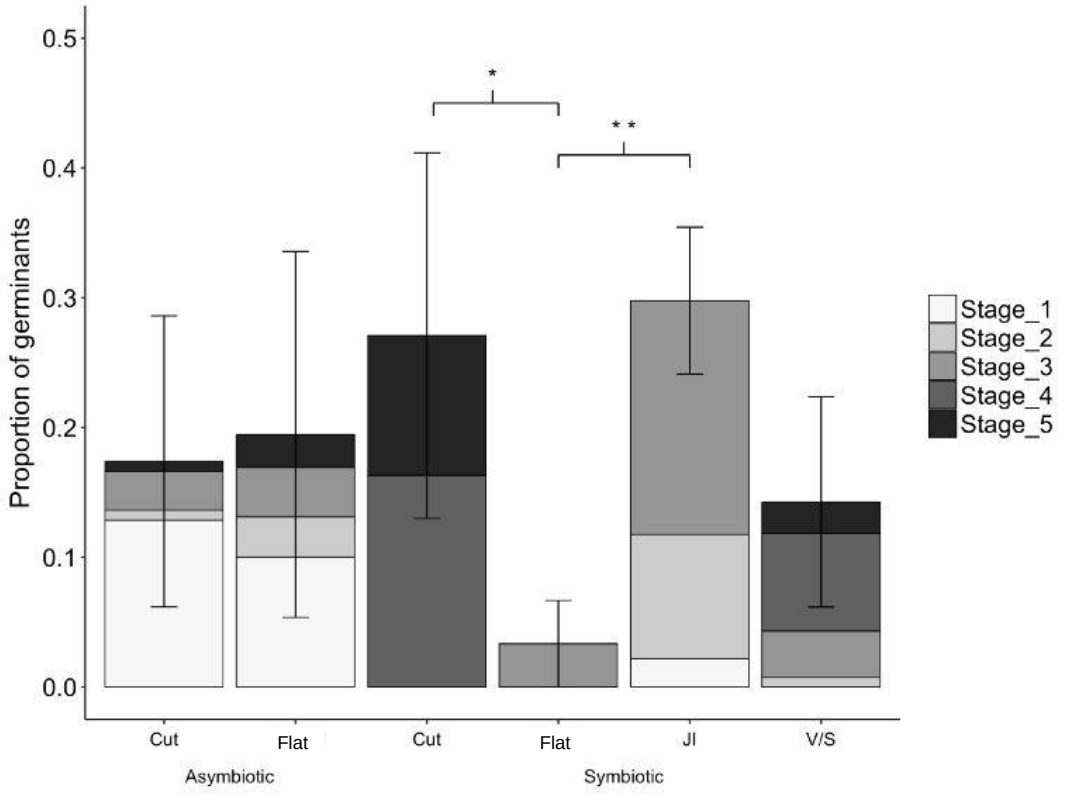

Figure 2. Mean numbers of germinated seed at different development stages for *Anacamptis morio* on asymbiotic Malmgren or H1 symbiotic media with B1 fungus on the following substrates: Cut (cut sections of cut agar based media); Flat (uncut agar based media); JI (John Innes No.1 + liquid media); V/S (Vermiculite and horticultural sand + liquid media) at 20 weeks from sowing. Development stage 1 (white); stage 2 (light grey); stage 3 (grey); stage 4 (dark grey); stage 5 (black). Error bars represent the standard error. * denote significant difference of <0.05; ** denote significant difference of <0.01; *** denote significant difference of <0.001.

Table 1. Protocorm development and germination of *Anacamptis morio* and *Dactylorhiza purpurella* at 10 weeks from sowing on asymbiotic Malmgren or H1 symbiotic media with B1 fungus on the following substrates: Cut (cut agar based media); Uncut (uncut agar based media); JI (John Innes No.1 + liquid media); V/S (Vermiculite and horticultural sand + liquid media). Numbers in parenthesis represent standard error of the data.

Species	Treatment		Mean % in developmental stage					Mean % Germination	
		In treatment	Stage 1	Stage 2	Stage 3	Stage 4	Stage 5	In treatment	Overall
Anacamptis morio	H1+B1 cut	Uncut	0	3.3 (3.3)	0	0	0	3.3 (3.3)	9.5 (3.5)
		Cut	0	0	12.5 (9)	7.6 (4)	6.9 (4.8)	27.1 (8.8)	
	H1+B1 flat	-	1.3 (1)	1.8 (1.3)	2.1 (1.1)	6.5 (2.9)	6.6 (2.5)	-	18.3 (3.8)
	JI & H1+B1	-	3.3 (1.3)	13.8 (2.5)	10.0 (2.6)	0	0	-	27.1 (3.7)
	V/S & H1+B1	-	0	0	9.2 (3.8)	3.4 (1.9)	0	-	12.6 (5.1)
	M+PP cut	Uncut	2.5 (1.9)	6.6 (2.3)	2.7 (2.6)	0	0	11.8 (4.3)	14.5 (2.4)
		Cut	12.1 (7.7)	3.1 (2)	0.7 (0.7)	0	0	15.8 (7.6)	
	M+PP flat	-	1.9 (1.3)	4.1 (1.8)	2.2 (1.3)	0	0	-	8.2 (2.7)
Dactylorhiza purpurella	H1+B1 cut	Uncut	3.9 (1.4)	4.8 (1.3)	27.6 (3)	0	0	36.3 (3.7)	42.8 (3.6)
		Cut	0.7 (0.7)	3 (1.1)	50.8 (4.9)	0	0	54.5 (5.4)	
	H1+B1 flat	-	1.1 (0.6)	2.8 (0.8)	30.8 (3.3)	0	0	-	34.8 (3)
	JI & H1+B1	-	0.4 (0.4)	4.1 (1.4)	8.6 (4.6)	0	0	-	13 (4.2)
	V/S & H1+B1	-	0	2.7 (1.2)	28.6 (6.4)	0	0	-	31.3 (6.5)
	M+PP cut	Uncut	4.9 (0.9)	10 (0.8)	0	0	0	15 (1.1)	15.6 (1)
		Cut	4.8 (1.3)	12.5 (1.8)	0	0	0	17.3 (2.4)	
	M+PP flat	-	4.5 (1.1)	8.9 (2.1)	0	0	0	-	13.3 (3.1)
	JI & M+PP	-	2.1 (0.8)	4.3 (1.1)	0.6 (0.6)	0	0	-	7 (1.4)
	V/S & M+PP	-	0.6 (0.6)	0.9 (0.4)	0	0	0	-	1.5 (0.6)

Table 2. Protocorm development and germination of *Anacamptis morio* and *Dactylorhiza purpurella* at 20 weeks from sowing on asymbiotic Malmgren or H1 symbiotic media with B1 fungus on the following substrates: Cut (cut agar based media); Uncut (uncut agar based media); JI (John Innes No.1 + liquid media); V/S (Vermiculite and horticultural sand + liquid media). Numbers in parenthesis represent standard error of the data.

Species	Treatment		Mean % in developmental stage					Mean % Germination	
		In treatment	Stage 1	Stage 2	Stage 3	Stage 4	Stage 5	In treatment	Overall
Anacamptis morio	H1+B1 cut	Uncut	0	0	3.3 (3.3)	0	0	3.3 (3.3)	9.5 (3.5)
		Cut	0	0	0	16.3 (8.9)	10.8 (5.1)	27.1 (8.8)	
	H1+B1 flat	-	1.1 (1)	1.9 (1.4)	0.3 (0.3)	0.4 (0.4)	14.6 (3.1)	-	18.3 (3.8)
	JI & H1+B1	-	2.2 (1.2)	9.6 (2.2)	18 (2.2)	0	0	-	29.8 (3.4)
	V/S & H1+B1	-	0	0.7 (0.7)	3.6 (2)	7.5 (3.7)	2.4 (1.7)	-	14.3 (6.1)
	M+PP cut	Uncut	10 (7.7)	3.1 (1.7)	3.8 (2.1)	0	2.6 (2.6)	19.5 (7.9)	16.1 (2.8)
		Cut	12.9 (7.7)	0.8 (0.8)	3 (2)	0	0.8 (0.8)	17.4 (7.5)	
	M+PP flat	-	1.9 (1.3)	2.9 (1.3)	3.4 (1.4)	0	0	-	8.2 (2.7)
Dactylorhiza purpurella	H1+B1 cut	Uncut	3.7 (1.7)	3.1 (0.7)	30.2 (3.2)	0	0	36.9 (3.6)	43.3 (3.6)
		Cut	0.7 (0.7)	2.5 (1.1)	51.3 (5)	0	0.3 (0.3)	54.7 (5.5)	
	H1+B1 flat	-	0.9 (0.6)	2.3 (0.7)	32.2 (3.5)	0	0.5 (0.5)	-	35.8 (2.9)
	JI & H1+B1	-	0.4 (0.4)	3.6 (1.2)	9.1 (4.5)	0	0	-	13.0 (4.2)
	V/S_&_H1+B1	-	0	2.2 (1.2)	29.9 (6.4)	0	0	-	32.1 (6.5)
	M+PP_cut	Uncut	1 .7 (0.8)	5.8 (0.9)	7.3 (0.8)	0	0	14.8 (1)	17.2 (1.3)
		Cut	0.9 (0.6)	9.9 (2.5)	14.2 (2)	0	0	25 (3)	
	M+PP_flat	-	3.4 (1.1)	5.4 (1.3	4.3 (1.2)	0	0	-	13.1 (3.1)
	JI & M+PP	-	2.1 (0.8)	4.3 (1.1)	0.6 (0.6)	0	0	-	7 (1.4)
	V/S_&_M+PP	-	0.6 (0.6)	1 (0.4)	0	0	0	-	1.5 (0.6)

After 20 weeks, development in *D. purpurella* had progressed as far as stage 3 with a few germinants reaching stage 5; three germinants on one plate of the H1+B1 flat and one germinant on H1+B1 cut treatment. All treatments had germinants reaching as far as stage 3 with exception to V/S M+PP which only developed as far as stage 2 (see Table 1 for week 10; Table 2 for week 20 developmental data). A small degree of protocorm death was observed on asymbiotic flat and uncut sections of cut plates after 20 weeks culture. There was a considerably higher proportion of more advanced germinants at stage 3 on both cut asymbiotic and cut symbiotic sections compared to their respective uncut sections. The symbiotic method gave far higher proportions of more developed germinants at stage 3 across all treatments when compared to respective asymbiotic treatments, with cut, flat and V/S having equally high proportions of germinants at stage 3 (Figure 1; 3).

Figure 3. Stages of development at 20 weeks from sowing: **a**) *Dactylorhiza purpurella* protocorm at stage 3 on H1+B1 cut (cut agar based H1 media with B1 fungus), displaying a leaf primordium (scale bar = 5 mm). **b**) *Dactylorhiza purpurella* protocorm at stage 3 on JI H1+B1 (John Innes No. 1 with H1 liquid media and B1 fungus, showing leaf primordium (scale bar = 2 mm). **c**) *Dactylorhiza purpurella* protocorms at stage 3 on V/S H1+B1 (Vermiculite and horticultural sand with H1 liquid media and B1 fungus, showing leaf primordium (scale bar = 2 mm).

Development in *A. morio* at 20 weeks was more advanced than *D. purpurella* with a higher proportion of germinants at stage 4 and 5 on both symbiotic and asymbiotic treatments. There was not a large difference in germination between asymbiotic cut and uncut sections of cut treatments, but there was a higher proportion of stage 2, 3 and 5 on the asymbiotic uncut

sections. Germinants on treatment M+PP flat, however did not develop beyond stage 3. A notable difference was observed in development between symbiotic cut and uncut sections with most germinants at stage 4 and 5 in the cut sections compared to germinants at stage 3 in uncut sections. In symbiotic treatments, JI had the highest germination and the largest proportion of germinants were at stage 2 and 3, none developed further. Compared to V/S which had lower germination but the highest proportion at stage 4 and a lower proportion at stage 5. The cut sections had higher germination than uncut sections, flat and V/S but lower than JI, with all germinants at advanced developmental stages 4 and 5. The flat treatment, however, has the highest proportion of germinants at stage 5 (see Table 1 for week 10 and Table 2 for week 20 developmental data). Due to the lack of seed, it should be noted that there was a comparably lower average number of seeds per plate in *A. morio* treatments at 11.66 (SE = 1.06) compared to *D. purpurella* at 61.32 (SE = 5.60). This may have contributed to the unclear picture given by *A. morio*, especially in the cut treatment (Figure 2; 3).

Statistical analysis

Rugosity treatments

In *D. purpurea*, an effect of rugosity treatment was observed, with a higher proportion of germination in the cut sections. This effect was significant in both symbiotic ($p = <0.001$) and asymbiotic methods ($p = <0.001$), and overall ($p = <0.001$; Appendix Table 1.1.a; Figure 5). The effect of culture method was also highly significant ($p = <0.001$), with higher germination in the symbiotic rather than the asymbiotic method in both the cut ($p = <0.001$) and uncut sections ($p = <0.001$; Appendix Table 1.2.a). There was a significant effect of the interaction between rugosity treatment and symbiotic method ($p = 0.0026$), but not with the asymbiotic method ($p = 0.0565$; Appendix Table 1.3.a; Figure 4; 5). In *A. morio*, there was a highly significant effect of rugosity with the symbiotic method ($p = <0.001$; Appendix Table 1.1.b; Figure 5). This corresponds to 1 germinant out of 94 seeds in the uncut sections across all replicates in this treatment compared to 8 germinants out of 38 seeds in the cut sections across all replicates in the treatment. For *A. morio*, no effect of rugosity in the asymbiotic media was observed ($p = 1$; Appendix Table 1.1.b; Figure 5). The effect of culture method was not significant overall ($p = 1$), with cut sections showing no significant

difference between symbiotic and asymbiotic media (p = 0.2371). However, there was significantly higher germination in the asymbiotic uncut compared to symbiotic uncut (p = 0.0029; Appendix Table 1.2.b). There was a significant effect of the interaction between rugosity treatment and symbiotic method (p = 0.0039), but similarly to *D. purpurella*, not for the asymbiotic method (p = 0.5925; Appendix Table 1.3.b; Figure 4; 5).

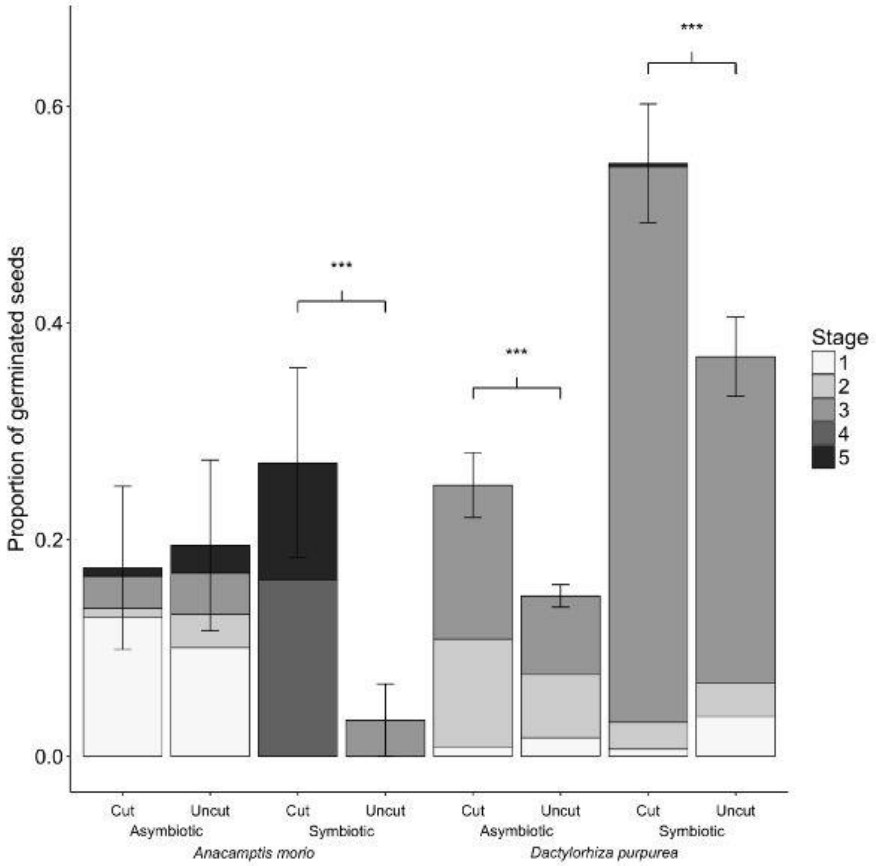

Figure 4. Mean numbers of germinated seed at different development stages for *Anacamptis morio* and *Dactylorhiza purpurella* on cut and uncut sections of cut treatments of agar based asymbiotic Malmgren media and symbiotic H1 media with B1 fungus at 20 weeks from sowing. Development stage 1 (white); stage 2 (light grey); stage 3 (grey); stage 4 (dark grey); stage 5 (black). Error bars represent the standard error. *** denote significant difference of <0.001.

Negative control (treatments)

Comparisons of flat plates vs uncut sections of cut plates showed that there was no effect of seeds being on a cut plate if they were not in a cut, with exception to

the symbiotic *A. morio* treatment. In *D. purpurea*, there was no effect of rugosity treatment on the proportion of germination on uncut sections in symbiotic (p = 0.74) or asymbiotic media (p = 0.44), and overall (p = 0.78; Appendix Table 2.1.a; Figure 5). In *A. morio* there was no effect of rugosity treatment on the proportion of germination on uncut sections in the asymbiotic method (p = 0.07), though there was a significant effect in the symbiotic method (p = <0.001); Appendix Table 2.1.b; Figure 5). The latter may be explained by the lack of seed in symbiotic uncut sections.

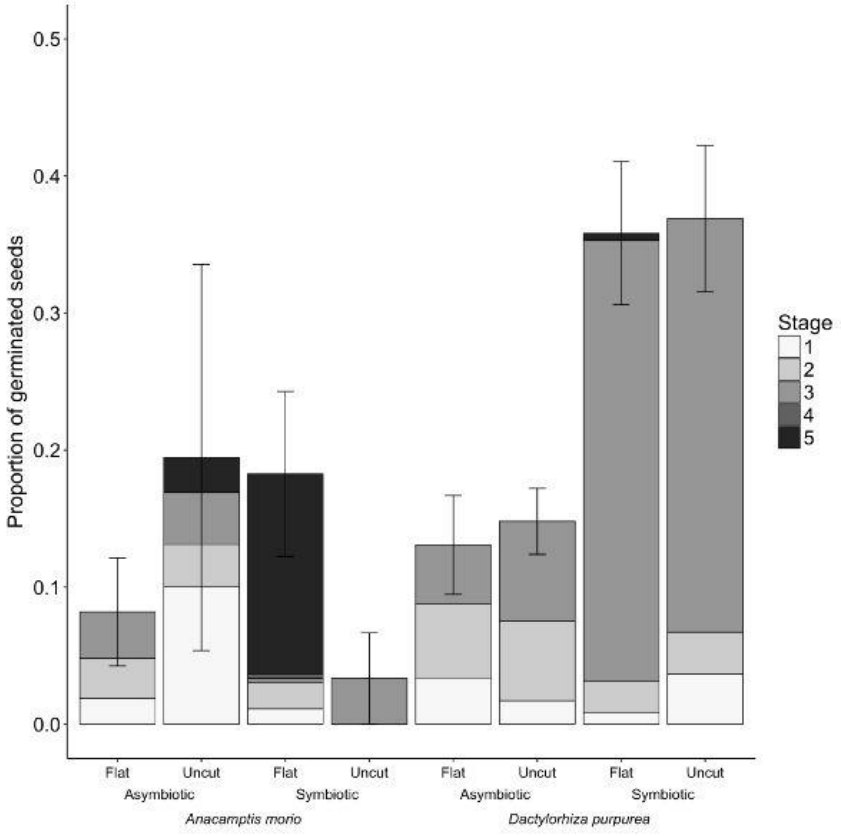

Figure 5. Negative control showing mean numbers of germinated seed at different development stages for *Anacamptis morio* and *Dactylorhiza purpurella* on uncut sections of cut treatments and flat treatments with agar based asymbiotic Malmgren media and symbiotic H1 media with B1 fungus at 20 weeks from sowing. Development stage 1 (white); stage 2 (light grey); stage 3 (grey); stage 4 (dark grey); stage 5 (black). Error bars represent the standard error.

Substrate complexity

Dactylorhiza purpurea showed a highly significant response to substrate complexity upon germination in asymbiotic (F = 33.69, df = 3, p = <0.001) and

symbiotic treatments (F = 11.38, df = 3, p = <0.001). In *A. morio*, a significant response to substrate complexity was observed in germination on symbiotic treatments (F = 5.07, df = 3, p = 0.0039). Analysis could not be performed on asymbiotic *A. morio* treatments as there was no germination on JI and V/S treatments. Pairwise comparisons between the different substrates are shown in Appendix Table 3.1.a; Figure 1 for *D. purpurella* and Appendix Table 3.1.b; Figure 2 for *A. morio*. Briefly, for *D. purpurella* with the asymbiotic method, the cut sections of the cut treatment gave the best germination and all substrates showed significant differences between them, with V/S giving the worst germination. In the symbiotic method, the cut sections of the cut treatment gave the best germination with JI being the worst germinator (Appendix Table 3.1.a; Figure 1). In *A. morio* symbiotic treatments, JI followed by the cut sections of the cut treatment were the most effective germinators, with the uncut sections of the cut treatment giving the lowest germination (Appendix Table 3.1.b; Figure 2).

Discussion

The hypothesis that increased substrate rugosity would have a positive effect on symbiotic germination and development in *D. purpurella* proved to be correct, with a significantly higher proportion of germination in the cut sections. However, this effect was also observed in asymbiotic cultures of *D. purpurella*. These observations were also supported by the controls, which showed that there was no effect of seeds being on a cut plate if they were not in a cut. The effect of the culture method was also highly significant in *D. purpurella* with a higher germination in symbiotic treatments in both cut and uncut sections when compared with asymbiotic treatments. In *D. purpurella* there was also a significant effect of the interaction between rugosity treatment and the symbiotic method. The presence of a symbiont increased the magnitude of germination overall, but when there were cuts in the media this effect was magnified. This suggests that the magnitude of the rugosity effect, depends on whether a symbiont is present with *D. purpurella*.

In symbiotic cultures of *A. morio*, while there was a significantly higher proportion of germination in the cut sections compared to the uncut sections of the cut plates, there was also higher germination on the flat plates compared to

the uncut sections of the cut plates, making it difficult to accept or reject the rugosity hypothesis unequivocally. The relatively low average number of seed for *A. morio* per plate (11.66) may have contributed to the uncertainty of these results. The asymbiotic method did not show a significant effect of rugosity for *A. morio*. The effect of the culture method was not significant although there was higher germination in the asymbiotic uncut compared to symbiotic uncut sections. There was a significant interaction between rugosity treatment and the symbiotic method, where germination on the symbiotic plates occurred almost exclusively in the cuts, while germination on the asymbiotic plates was independent of the presence of cuts. Although these results are harder to interpret, there does seem to be evidence of an effect of rugosity increasing germination, but only in the symbiotic cultures. As the two species tested are in the same sub-tribe (Orchidinae) it was expected that they would respond in a similar manner to the different treatments. However, this was clearly not the case and clear differences among the species were observed. This may have been due to interspecies differences in seeds or an issue with seed quality or sterilisation. Further investigation may be necessary to confirm these results.

Dactylorhiza purpurella showed a significant response to substrate complexity with the asymbiotic and symbiotic method. Symbiotic cut sections and JI showed significant differences in germination when compared to other symbiotic treatments, except with V/S. Cut sections followed by uncut sections of cut treatments showed the highest germination, with JI having the least. As with the symbiotic method, asymbiotic cut sections followed by uncut sections of cut treatments showed higher germination than JI, with V/S giving the worst germination. This may indicate that on the JI and V/S media, nutrient availability was not optimal to induce effective germination in asymbiotic cultures. Although orchid seed is capable of germinating in water, further development is highly reliant on the provision of suitable nutrients on an asymbiotic media or through association with an appropriate fungus (Rasmussen, 2006). Mycorrhizal fungi have been shown to mobilise and exchange nutrients with protocorms (Kuga *et al*. 2014), seedlings and adult plants (Cameron *et al*., 2006; 2007). This suggests that there was either a need for a higher nutrient concentration or a need for symbiotic fungi to mobilise nutrients for germinants on a complex substrate.

In *A. morio,* a significant effect of substrate complexity was observed upon germination in symbiotic cultures. Unlike *D. purpurella*, germination in *A. morio* was highest on JI followed by cut sections of the cut treatments. These treatments were significantly higher than uncut sections of cut treatments which gave the lowest germination. There was no germination at all in the asymbiotic V/S and JI treatments. These results similarly suggest that there was a deficiency or a negative factor affecting germination on non-agar based media, and that fungal symbionts enhance germination on complex substrates. The lower germination that was observed on symbiotic JI treatments may be a result of excessive nutrients, as John Innes No. 1 contains a mixture of organic and inorganic fertilisers (John Innes Manufacturers' Association, 2017). This in addition to the liquid nutrient media that was added may have resulted in elevated nutrients, altering fungal growth and potentially resulting in too high an osmolarity in the media, inhibiting germination (Rasmussen, 2006).

Development in *D. purpurella* generally progressed as far as stage 3, with a few germinants reaching stage 5 after 20 weeks culture on symbiotic agar-based media. Both asymbiotic and symbiotic cut sections had a considerably higher proportion of germinants at stage 3 in comparison to respective uncut sections. The symbiotic method, however, gave far higher proportions of more developed germinants at stage 3 across all treatments when compared to respective asymbiotic treatments. Symbiotic cut and uncut sections of cut treatments, flat or V/S treatments, all produced large numbers of germinants at stage 3. The additional time and effort of preparing the sterile V/S media plates would make this prohibitive when compared to the simpler preparation of the H1 agar based media. With the highest germination and development up to stage 5, the symbiotic cut treatment would prove most suitable for propagating *D. purpurella.* However, if the V/S H1 media was altered to a larger scale *ex vitro* using pots or culture trays in a similar manner to Quay *et al*., (1995) or Batty *et al*., (2006a), it may prove to be a more efficient symbiotic propagation method.

Although it has been shown that increased substrate rugosity can have a positive effect on germination in symbiotic cultures of *A. morio* and *D. purpurella*, it was also observed that a positive effect of increased rugosity in asymbiotic cultures of *D. purpurella*. The increased rugosity of cut agar as well as increased texture in inorganic or soil based substrates provide higher surface areas. This

could allow for higher absorption of moisture and nutrients which may have had a positive effect on germination in both asymbiotic and symbiotic cultures. A small amount of protocorm death occurred in *D. purpurella* on asymbiotic flat and uncut sections of cut plates after 20 weeks culture. Protocorm death on the asymbiotic media could be a result of the build-up of waste products such as phenolic compounds (Van Waes, 1987; Rasmussen, 2006). Interestingly there was no death in the cut sections, JI or V/S media, perhaps suggesting that the increased surface area of the cracks and particles allowed for better access to absorptive agents such as charcoal in the media and/or provided a greater ability for toxin dispersal.

Anacamptis morio reached a more advanced state of development than *D. purpurella* at 20 weeks culture with stage 4 and 5 germinants on asymbiotic and symbiotic treatments. Asymbiotic uncut sections had the highest proportions of advanced germinants. Whereas in symbiotic culture, cut sections had the highest proportion of advanced germinants. Germination was highest in the symbiotic JI treatment, but development had not progressed beyond stage 3, suggesting a potential deficiency of nutrients in the media or that the larger number of symbiotic germinants may have begun to exhaust the resources of the media, slowing development. There was a notable difference in development between symbiotic cut and uncut sections with most germinants at stage 4 and 5 in the cut sections and germinants only at stage 3 in the uncut sections. The symbiotic flat treatment had the largest proportion of stage 5 germinants. This treatment along with symbiotic cut sections and V/S gave the highest proportion of germinants at stage 4 and 5 and can be considered to be optimal methods for germination and development of *A. morio*. The flat B1 fungus symbiotic treatment has previously been shown to be the best treatment for germination and development of this species (Chapter 3) and as it requires less time and effort to prepare, would be the most suitable treatment for propagating this species.

From the initial observations made in symbiotic cultures of *O. apifera* in Chapter 4 it was thought that the rugosity of the media may have affected the growth of the fungus and enhanced its ability to germinate *O. apifera*. It has shown to be the case with *A. morio* and *D. purpurella*, and has been demonstrated that the magnitude of the rugosity effect depends on whether a symbiont is present and the species of orchid. Resultantly, increased substrate

rugosity combined with a suitable fungal symbiont can give higher germination, increasing the productivity of the symbiotic method. The observations of higher germination and development in symbiotic compared to asymbiotic treatments in *D. purpurella* concurs with observations of a symbiotic propagation advantage made with other terrestrial orchids: *A. morio; Dactylorhiza incarnata* (L.) Soó*,* (Chapter 3); *Anacamptis laxiflora* (Lam.) R. M. Bateman, Pridgeon & M. W. Chase; *O. apifera,* (Chapter 4); *Dactylorhiza majalis* (Rchb.)*,* (Rasmussen, 1990a); *Spiranthes magnicamporum* Sheviak, (Anderson, 1991). For *ex situ* or commercial propagation such results, however, are reliant on the additional time investment of obtaining and maintaining suitable fungi.

Despite the broad spectrum of existing literature on asymbiotic and symbiotic propagation of orchids, with many investigators using a range of different media and substrates (Clements *et al*., 1986; Muir, 1989; Quay *et al*., 1995; Batty *et al.,* 2006b; Rasmussen, 2006; Yagame *et al*., 2007; Dutra *et al*., 2008; Aewsakul *et al*., 2013). To my knowledge there are no studies focussing on the effects of substrate complexity, rugosity and texture on the germination and development of orchids. Rasmussen (2006), states that soils in orchid localities often tend to be porous with a well-developed crumb structure with orchid endophytes infecting roots in the top soil. This suggests that orchid endophytes prefer well aerated soils. In a study testing germination with *ex situ* and *in situ* seed packets, Brundrett *et al*., (2003) found that greatest fungal activity, orchid seed germination and development most consistently occurred in coarse soil organic matter in topsoils and litter layers. Fine organic matter and bulk soil on the other hand, were generally ineffective reservoirs of fungal inoculum. This is supported by the findings in this study in non-agar based symbiotic cultures of *D. purpurella*. Seeds sown on the larger grained V/S substrate showed higher germination and a greater proportion of germinants at the most advanced development stages when compared to the finer grained JI. The results of *A. morio* also somewhat support this, despite a lower germination on V/S compared to JI there was more advanced development of germinants on V/S. It should be noted that observations *in vitro* cannot necessarily be directly compared to those in nature and if doing so they should be interpreted with due caution (Rasmussen, 2006).

Hyphal tip growth of filamentous fungi such as *Rhizoctonia solani* Kühn, primarily follow straight lines but can make small changes in direction at random points. Formation of new hyphae via branching occurs at acute angles to the growth (Dix & Webster, 1995; Boswell *et al*., 2007). This branching architecture and filamentous growth allows for effective colonisation of soil and other complex substrates. Considering that horizontal agar plates are quite different to the three dimensional structure of the soil and plant root environment that the fungus would naturally colonise. It may be that the morphology or growth form of fungi growing on flat media is not identical to its native state. The study shows that optimal symbiotic germination and development occurred in cuts of agar-based media and the larger grained inorganic V/S treatment. Providing substrates with a higher substrate complexity may help optimise fungal growth for symbiotic interactions between orchid and fungus. Further research is needed to investigate if this is the case.

Studies by Scott & Carey, 2002; Diez, 2007 have shown enhancing effects of moisture on symbiotic germination using seed packets in field studies with terrestrial orchids. Trembley *et al*., (1998) and Zettler *et al*., (2011) have also shown that substrate moisture levels and presence of moisture trapping organisms such as mosses are key to germination of epiphytic orchids *in situ*. Rasmussen *et al*., (2015) states that it is unclear whether these results are a direct result of moisture aiding physical imbibition of orchid seeds or indirectly due to the presence and enhanced growth of mycorrhizal fungi. Yoder *et al*., (2000) demonstrated that the presence of mycorrhizal fungi enhanced germination by increasing water uptake capacity in orchid seeds and seedlings, and may account for the observations of higher germination and/or development in symbiotic cultures in this study.

The higher surface areas found in cut agar-based and inorganic substrates may have led to increased connections or chances of connections between seeds and fungal hyphae in symbiotic cultures. Increased aggregate size, lower bulk soil densities (Otten *et al.,* 2001) and increased soil porosity (Otten *et al.,* 2004) have been shown to aid the spread of mycelia through soils. This may explain the higher germination and proportion of advanced germinants found on V/S compared to JI substrates with *D. purpurella*. The opposite was observed in germination with *A. morio,* though development was far more advanced on the

V/S treatment, perhaps suggesting the potentially increased mycelial spread and connections allowed for greater mobilisation of water and nutrients for developing germinants on the V/S media.

This study provides a gradation of substrate complexity from a simple flat agar surface to a more complex small particle soil based medium and provides some insight into the effects that influence germination in orchid seeds on natural soils and other surfaces. The results show that substrate rugosity is a significant factor in the germination of these terrestrial orchids *in vitro*. Considering that orchids will germinate on a variety of substrates in nature with varying rugosity and complexities; soils, bark and moss (Trembley *et al*., 1998; Rasmussen, 2006; Zettler *et al*., 2011). It is evident that substrate complexity, rugosity and texture is an important factor in orchid germination and development. Whether it is an effect of substrate complexity on the fungus or a direct effect of substrate or both depends on the species and suggests further research is needed to help better determine its implications in orchid-fungal interactions, their symbiosis and orchid propagation.

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Appendix

Analysis 1. Difference between cut and uncut sections inside cut agar based treatments.

Randomization test (n=100,000).

Appendix Table 1.1.a *Dactylorhiza purpurea*

Proportion of germinants in cut and uncut sections with asymbiotic and symbiotic methods.

	Cut	Uncut
Asymbiotic	0.2503	0.1480
Symbiotic	0.5474	0.3688

Rugosity, for each method and overall

Asymbiotic (M+PP)

	Cut	Uncut	Difference
Observed % germination	0.2503	0.148	0.1023
Simulated % germination	0.1993	0.1992	0.0209
P-value	<0.001 ***	<0.001 ***	<0.001 ***

Symbiotic (H1+B1)

	Cut	Uncut	Difference
Observed % germination	0.5474	0.3688	0.1786
Simulated % germination	0.458	0.4582	0.0411 *
P-value	0.0052 **	0.0047 **	<0.001 ***

Overall

	Cut	Uncut	Difference
Observed % germination	0.3989	0.2584	0.1405
Simulated % germination	0.3287	0.3287	0.0298 *
P-value	0.0032 **	0.0029 **	<0.001 ***

Summary (cut vs uncut)

	Asymbiotic	Symbiotic	Overall
Difference	0.1023	0.1786	0.1405
P-value	<0.001 ***	<0.001 ***	<0.001 ***

Appendix Table 1.1.b *Anacamptis morio*

Proportion of germinants in cut and uncut sections with asymbiotic and symbiotic methods***.***

	Cut	Uncut
Asymbiotic	0.1739	0.1947
Symbiotic	0.2708	0.0333

Rugosity for each method

Asymbiotic (M+PP)

	Cut	Uncut	Difference
Observed % germination	0.1739	0.1947	-0.0208
Simulated % germination	0.1844	0.1842	0.0611
P-value	0.5806	0.5806	1

Symbiotic (H1+B1)

	Cut	Uncut	Difference
Observed % germination	0.2708	0.0333	0.2375
Simulated % germination	0.139	0.1387	0.0557
P-value	0.0046 **	0.0048 **	<0.001 ***

Note: There was only one replicate with one germinated seed in the uncut sections.

Overall

	Cut	Uncut	Difference
Observed % germination	0.2204	0.1083	0.1122
Simulated % germination	0.161	0.1613	0.0406
P-value	0.061	0.0606	0.024 *

Summary (cut vs uncut)

	Asymbiotic	Symbiotic	Overall
Difference	-0.0208	0.2375	0.1122
P-value	1	<0.001 ***	0.024 *

Appendix Table 1.2.a *Dactylorhiza purpurea*

Method, for each rugosity type and overall.

Cut

	Asymbiotic	Symbiotic	Difference
Observed % germination	0.2503	0.5474	-0.2971
Simulated % germination	0.3987	0.3987	0.0469
P-value	1	1	<0.001 ***

Uncut

	Asymbiotic	Symbiotic	Difference
Observed % germination	0.148	0.3688	-0.2208
Simulated % germination	0.2583	0.2585	0.0313
P-value	1	1	<0.001 ***

Overall

	Asymbiotic	Symbiotic	Difference
Observed % germination	0.1992	0.4581	0.2589
Simulated % germination	0.3286	0.3286	0.0297
P-value	1	1	<0.001 ***

Summary (Symbiotic vs Asymbiotic)

	Cut	Uncut	Overall
Difference	-0.2971	-0.2208	0.2589
P-value	<0.001 ***	<0.001 ***	<0.001 ***

Appendix Table 1.2.b *Anacamptis morio*

Method, for each rugosity type and overall.

Cut

	Asymbiotic	Symbiotic	Difference
Observed % germination	0.1739	0.2708	-0.097
Simulated % germination	0.2202	0.2204	0.0649
P-value	0.791	0.7931	0.2371

Uncut

	Asymbiotic	Symbiotic	Difference
Observed % germination	0.1947	0.0333	0.1614
Simulated % germination	0.1083	0.108	0.0496
P-value	0.0264 *	0.0262 *	0.0029 **

Overall

	Asymbiotic	Symbiotic	Difference
Observed % germination	0.1843	0.1389	-0.0454
Simulated % germination	0.1611	0.1612	0.0407
P-value	0.2768	0.2612	1

Summary

	Cut	Uncut	Overall
Difference	-0.097	0.1614	-0.0454
P-value	0.2371	0.0029 **	1

Table 1.3. Interaction, the effect of rugosity (cut or uncut) on the different methods (symbiotic or asymbiotic). The difference between cut and uncut sections in each method was calculated, showing how much rugosity increases germination. Then the difference in this increase between the methods was calculated. This is how strong the effect of rugosity is in each method i.e. it is the magnitude of the effect of cut vs uncut treatments having corrected for asymbiotic or symbiotic method.

Appendix Table 1.3.a *Dactylorhiza purpurea*

	Asymbiotic	**Symbiotic**	**Difference**
Observed mean %	0.1023	0.1786	0.0763
Simulated mean %	-0.0002	0.0002	0.073
P-value	0.0565	0.0026 **	0.4057

Appendix Table 1.3.b *Anacamptis morio*

	Asymbiotic	**Symbiotic**	**Difference**
Observed mean %	-0.0208	0.2375	0.2583
Simulated mean %	-0.00016	-0.00001	0.0994
P-value	0.5925	0.0039 **	0.0373 *

Analysis 2. Difference between uncut sections of cut treatments and uncut treatments (control).

Randomization test (n=100,000).

Appendix Table 2.1.a *Dactylorhiza purpurea*

Asymbiotic (M+PP)

	Uncut treatment	Uncut sections	Difference
Observed % germination	0.1480	0.1308	0.0172
Simulated % germination	0.1400	0.1401	0.0176
P-value	0.3274	0.7226	0.4457

Symbiotic (H1+B1)

	Uncut treatment	Uncut sections	Difference
Observed % germination	0.3688	0.3583	0.0105
Simulated % germination	0.3636	0.3635	0.0259
P-value	0.4122	0.5873	0.7474

Appendix Table 2.1.b *Anacamptis morio*

Asymbiotic (M+PP)

	Uncut treatment	Uncut sections	Difference
Observed % germination	0.1947	0.0820	0.1127
Simulated % germination	0.1429	0.1433	0.0524
P-value	0.1810	0.9234	0.0775

Symbiotic (H1+B1)

	Uncut treatment	Uncut sections	Difference
Observed % germination	0.3333	0.1826	0.1493
Simulated % germination	0.1081	0.1081	0.0325
P-value	0.9983	0.002 **	<0.001 ***

Note: There was only one replicate with one germinated seed in the uncut sections.

Analysis 3. Comparison across multiple substrates for each species. PERMANOVA analysis with 1000 permutations and a post-hoc analysis using pairwise comparisons between the different substrates. P values adjusted with Bonferroni correction for multiple comparisons.

Appendix Table 3.1.a *Dactylorhiza purpurea*

Asymbiotic (M+PP)

Comparisons	F	*P*
M+PP cut - M+PP uncut	10.3798	<0.05 *
M+PP cut - JI & M+PP	29.9298	<0.01 **
M+PP cut - V/S & M+PP	58.6324	<0.01 **
M+PP uncut - JI & M+PP	20.7500	<0.01 **
M+PP uncut - V/S & M+PP	118.8814	<0.01 **
JI & M+PP - V/S & M+PP	13.0818	<0.05 *

Symbiotic (H1 + B1)

Comparisons	F	*P*
H1+B1 cut - H1+B1 uncut	7.3813	<0.05 *
H1+B1 cut – JI & H1+B1	36.2648	<0.01 **
H1+B1 cut - V/S & H1+B1	7.1346	0.0719
H1+B1 uncut – JI & H1+B1	18.1578	<0.05 *
H1+B1 uncut - V/S & H1+B1	0.4146	1
JI & H1+B1 - V/S & H1+B1	6.055	0.1319

Appendix Table 3.1.b *Anacamptis morio*.

Symbiotic (H1+B1)

Comparisons	F	*P*
H1+B1 cut - H1+B1 uncut	7.5271	<0.05 *
H1+B1 cut – JI & H1+B1	0.0956	1
H1+B1 cut - V/S & H1+B1	1.5358	1
H1+B1 uncut – JI & H1+B1	30.4763	<0.01 **
H1+B1 uncut - V/S & H1+B1	2.5008	0.6953
JI & H1+B1 - V/S & H1+B1	4.9681	0.2038

Chapter 6.

Asymbiotic and symbiotic germination of five epiphytic orchids from the subtribe Pleurothallidinae - advantage for initial symbiotic germination

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Abstract

The orchid subtribe Pleurothallidinae is likely the most species rich of all orchid subtribes with more than 5000 species found in the tropical Americas, and many having restricted distributions in niche habitats. Little literature exists on the propagation of these orchids. Germination and development comparisons were demonstrated utilising asymbiotic and symbiotic methods for six orchids from four genera within this subtribe: *Masdevallia melanoxantha* Linden & Rchb.f.; *Masdevallia menatoi* Luer & R.Vásquez*; Masdevallia patchicutzae* Luer & Hirtz*; Pleurothallopsis microptera* (Schltr.) Pridgeon & M.W.Chase*; Restrepia contorta* (Ruiz & Pav.) Luer; *Stelis sp*. Peru. All orchids were sown on half strength and in the case of *M. melanoxantha*, *M. menatoi* and *P. microptera,* full strength asymbiotic Knudson C, Sigma P6668 and Vacin and Went media. For *M. melanoxantha,* additional treatments containing 100 ml l^{-1} of coconut water were included with half and full strength asymbiotic media. Symbiotic sowings used H1 media with fungi isolated from orchid roots of this subtribe growing *ex situ* in the National Plant Collection® of Pleurothallid orchids, maintained by the North of England Zoological Society.

Germination amongst species was low on asymbiotic media (<12%) whereas two fungal isolates (Df from *Dracula felix* (Luer) Luer and Mm from *M. menatoi*) supported significantly higher germination at 14 - 53% with *M. melanoxantha, M. menatoi, M.* patchicutzae (only Mm tested), *R. contorta* (only Df tested), and *P. microptera*, with the fungal isolate Mm proving most successful. There was a significant overall effect of treatment on the species tested. Whereas asymbiotic media strength and coconut water did not have a significant effect on germination. Development on the symbiotic treatments and on the asymbiotic Knudson C treatment did not progress beyond early stage protocorms. Fully formed plantlets with leaves and roots were obtained after 20 weeks culture with *M. melanoxantha* on half strength Sigma P6668 with and without coconut, and with *P. microptera* on half and full strength Sigma P6668*.* The results of this study demonstrated an initial advantage for symbiotic germination, but that development progressed furthest on one of the asymbiotic media. These findings could assist with improving the production of orchid seedlings for conservation and horticulture.

Keywords Orchidaceae • Mycorrhizae • Germination • Propagation • Conservation

Introduction

The orchid subtribe Pleurothallidinae (Pleurothallid orchids) is likely to be the most species rich of all orchid subtribes, representing more than 5000 species of the currently accepted 28,484 species in the family Orchidaceae (Karremans, 2016, WCSP, 2017). The Pleurothallid orchids are Neotropical in distribution and can be found from Florida and Mexico in North America down through the Caribbean, Central and South America as far south as northern Argentina (Pridgeon, 1982, Müller *et al*., 2003). The description of the subtribe by Pridgeon *et al*., (2001) is particularly apt 'The majority are dipteran, deceit-pollinated epiphytes with sympodial growth, unifoliate nonpseudobulbous stems or "ramicauls," conduplicate leaves, velamentous roots, and an articulation between the pedicel and ovary'. The subtribe also contains a number of lithophytes, rheophytes and terrestrial species (Higgins & Williams, 2009). The species richness of Pleurothallidinae is greatest in the Andean cloud forests (Pridgeon, 1982) where there is high degree of endemism (Dodson, 2005; Higgins & Williams, 2009; Pérez-Escobar *et al*., 2017).

The diversity associated with the family Orchidaceae and its subtribe Pleurothallidinae has resulted in the development of specific relationships with insect pollinators (Borba *et al*., 2011; Barbosa *et al*., 2009) and mycorrhizal fungi (Suárez *et al*., 2016; Suárez & Kottke, 2016), which enable the colonisation of niche habitats. Pleurothallid orchids are largely characterized by narrowly restricted populations, found in niche habitats containing only small numbers of individuals (Tremblay & Ackerman, 2001; Jost, 2004; Crain & Tremblay, 2012, Pérez-Escobar *et al*., 2013; Pérez-Escobar *et al*., 2017). In such cases, even minor changes to their habitat can have a negative impact on their populations (Cribb *et al*., 2003; Swarts & Dixon, 2009b). As a result, a large number of these orchids are now considered rare, threatened or endangered (Llamozas *et al*., 2003; Calderón-Sáenz, 2006; Millner, 2013). In order to preserve these species and their diversity, active *in situ* conservation, and where necessary, *ex situ* conservation efforts are required. Therefore, the study and optimisation of *ex situ*

propagation methods is essential for preserving many rare species, especially where populations have become unviable due to severe habitat reduction, fragmentation or collection pressures (Swarts & Dixon, 2009b, Millner, 2013).

The specialisation found in Orchidaceae has resulted in a heavy reliance on mycorrhizal fungi for at least some stages in their life cycle, particularly during germination (Rasmussen, 2006). Although orchid seed is capable of germinating in water, they are highly reliant on being infected by an appropriate fungus for further development to occur (Rasmussen, 2006). Epiphytic orchids are not as reliant on mycorrhizal fungi as terrestrial orchids. This is due to the nature of their permanent green growth above ground and their ability to produce photosynthetic protocorms at an early stage (Knudson, 1929; Rasmussen, 2006). Despite this, fungi play a key role in the lifecycles of these orchids, from germinating seeds to developing seedlings and adult plants (Hadley, 1970; Cruz-Higareda *et al*., 2015; Suarez *et al*., 2016; Suarez & Kottke, 2016).

Orchids are commonly propagated from seed using axenic culture on agar based nutrient media. These can be symbiotic, where a symbiotic fungus is cultured in association with orchid seed or asymbiotic, where seeds are sown without fungus on a sterile nutrient media. Asymbiotic media contain the nutrients necessary to assist germination and development, replicating those that mycorrhizal fungi would normally provide in nature (Rasmussen, 2006; Seaton *et al.,* 2011). Symbiotic propagation methods commonly utilise a complex carbohydrate source such as oats (Clements & Ellyard, 1979) which is metabolised by the fungus and made available to orchid germinants through mycorrhizal association (Rasmussen, 2006). Asymbiotic methods are most commonly used in scientific investigation and commercial propagation of epiphytic orchids (Butcher & Marlow, 1989; Stenberg & Kane, 1998; Kauth *et al*., 2006; 2008a; Dutra *et al*., 2009; Seaton *et al.,* 2011). Symbiotic methods on the other hand have only seen notable use by conservationists and researchers (Zettler, 1997b; Zettler *et al*., 2007; Otero & Bayman, 2009; Nontachaiyapoom *et al.,* 2011; Seaton *et al.,* 2011; Hajong *et al*., 2013; Tan *et al*., 2014). For further details on media see: Chapter 3; 4; 5 and for further details on symbiotic culture see Chapter 2; 3; 4; 5.

Asymbiotic media strength is an important factor affecting orchid germination, certain media can prove to have too high an osmolarity for particular orchid species (Fast, 1978; Van Waes, 1984; Brundrett *et al*., 2001, Rasmussen, 2006). Some orchid species, such as those within the genus *Masdevallia* can display different preferences to media strength and formulation (Mckendrick, 2000). The addition of phytohormones have been shown to have a positive effect on orchid germination and development. Phytohormones can be included in asymbiotic media in the form of pure research-grade chemicals such as the cytokinins: kinetin, 6-benzylaminopurine (Harvais & Hadley, 1968; Harvais, 1982; Van Waes, 1984, Arditti, 2009); 6-(α,α-dimethylallylamino)-purine (De Pauw *et al*., 1995), and auxins: indoleacetic acid, indolebutric acid, naphthalenic acid (Van Waes, 1984; Arditti, 2009). These hormones can be added in a less accurate manner in the form of complex organic additives, including portions of plant tubers; boiled potato, seed extracts or fruit juices: pineapple juice, coconut milk (Malmgren, 1996; Kitsaki *et al.* 2004; Pierce *et al*., 2015), coconut water or mixtures of these such as banana homogenate (Butcher & Marlow, 1989; Arditti, 2009; Kaur & Bhutani, 2014). Coconut water has been widely utilised in asymbotic propagation (Oliva & Arditti, 1984; Lo *et al.*, 2004; Sharma *et al*., 2005; Arditti, 2009; Abbas *et al*., 2011; Abraham *et al.* 2012) and consists of the liquid endosperm of the coconut, *Cocos nucifera* L. It contains the phytohormones: zeatin, indoleacetic acid and a smaller proportion of kinetin as well as other substances that stimulate orchid growth, such as sugars, amino acids and vitamins (Yong *et al*. 2009). The phytohormones support cell division and rapid growth, and when combined with the other constituents can potentially give better germination and development than pure phytohormones on their own (Arditti, 2009; Yong *et al*. 2009; Pierce *et al*., 2015).

The symbiotic method has largely seen use in studies of terrestrial orchids (Harvais & Hadley, 1967a; 1967b; Hadley, 1970; Warcup, 1971; 1973; 1981; 1988; Clements & Ellyard, 1979; Clements *et al.*, 1986; Muir, 1989; Rasmussen, 2006) where there is some debate as to which is the best method of propagation. Asymbiotic propagation can produce large numbers of healthy plants without having to invest time and effort of isolating, maintaining and providing suitable conditions for fungal symbiosis (Rasmussen, 2006). Previous studies suggest that when combined with appropriate fungi, some terrestrial orchid's exhibit

higher germination, developmental *in vitro* and survival *ex vitro* when compared with asymbiotic methods (Muir, 1989; Anderson, 1991; Seaton *et al.,* 2011; Hughes, Chapter 3, 4).

Very little literature exists on the propagation of orchids of the subtribe Pleurothallidinae, but notable studies using asymbiotic techniques have focused on species of *Masdevallia* (Pedroza-Manrique, 2006; Lopez-Roberts, 2007; Clavijo, 2010) and *Restrepia* (Millner *et al.,* 2008, Millner, 2013). Few studies have investigated symbiotic propagation, however, Otero & Bayman, (2009) compared asymbiotic and symbiotic methods with the Pleurothallid orchid *Lepanthes rupestris* Stimpson in their research on epiphytic Puerto Rican orchids and they found no significant difference between asymbiotic and symbiotic germination for this species.

The aim of this study was to ascertain the most effective method for germination and development of six orchid species from the subtribe Pleurothallidindae by testing the suitability of variations of three asymbiotic media: Knudson C; Sigma P6668; Vacin and Went, at full and half strength and with and without coconut water. As well as three fungi isolated from roots of the Pleurothallid orchids: *Dracula felix* (Luer) Luer; *Lepanthopsis astrophora* (Rchb.f. ex Kraenzl.) Garay; *Masdevallia menatoi* Luer & R.Vásquez grown *ex situ* in the National Plant Collection® of Pleurothallid orchids held by Steve Manning and the North of England Zoological Society, Chester, UK..

It was hypothesised that lower media concentrations and the presence of coconut water in asymbiotic media would have a positive effect on germination and development in the Pleurothallid orchids tested. With the symbiotic fungi, it was hypothesised that different responses would be seen for each orchid-fungal combination, with some fungi inducing no germination, others inducing germination but not further development and some potentially inducing higher germination and more advanced development. This experiment was designed to inform the development of propagation methods for these orchids, which could be implemented in future conservation efforts and horticultural production.

Materials and Methods

Organismal details

Six orchid species from the subtribe Pleurothallidinae were used. (1) *Masdevallia melanoxantha* Linden & Rchb.f. is a medium to large epiphytic species with thickly coriaceous leaves and coarse roots (Gerritsen & Parsons, 2005). It is found in the north eastern Cordillera of Boyaca and Norte de Santander, Colombia and northwest Venezuela in cloud forests on mossy tree trunks and branches at 1400 – 2700 m. It is considered to be vulnerable on the Colombian (Calderoón-Sáenz, 2006) and Venezuelan plant red lists (Llamozas *et al*., 2003) and is threatened by habitat loss due to deforestation and agricultural expansion (Llamozas *et al*., 2003; Calderón-Sáenz, 2006). (2) *Masdevallia menatoi* is similar in growth form to *M. melanoxantha* but is smaller in size. It is found in cloud forests at 1800 m in the Province of Sud Yungas, Department of La Paz, Bolivia (Luer, 1980) and has been recorded as extinct in the wild (Walter & Gillett, 1998). (3) *Masdevallia patchicutzae* Luer & Hirtz is similar in growth morphology to *M. meantoi* and is found in cloud forests in southeastern Ecuador and Peru at 1000 – 1300 m (Dodson & Luer, 2009). (4) *Pleurothallopsis microptera* (Schltr.) Pridgeon & M.W.Chase is a small to medium sized epiphytic or terrestrial orchid with thickly coriaceous elliptical leaves and can be found in cloud forest in Colombia, Ecuador and Peru at 1700 – 2400 m (Luer, 1978; Pridgeon & Chase, 2001). (5) *Restrepia contorta* (Ruiz & Pav.) Luer is a small sized epiphyte, tufted in growth habit with erect ramicauls and elliptical, apically tridentate leaves. It is found in cloud forests in the northern Andes from Peru through to Ecuador, Colombia and north-western Venezuela at 1400 – 3500 m altitude (Luer & Escobar-Restrepo, 1996). This species has lost nearly half its known locations across its range and is considered to be vulnerable (Millner, 2013). It is noted as being of least concern on the Colombian plant red list (Calderón-Sáenz, 2006) and can be considered critically endangered in Ecuador, Peru, Venezuela (Millner, 2013). (6) An unknown species of *Stelis sp.* is a small to medium, presumably epiphytic species which was present in the collection with the notes: Peru at 1700 m altitude.

Three fungi were used in this study, and were isolated from roots of Pleurothallid orchids growing in the National Plant Collection® of Pleurothallid orchids. The fungi were given the abbreviations: Df which was isolated from

Dracula felix, an epiphytic orchid of cloud forests in Colombia and Ecuador at 1200 – 2500 m (Calderón-Sáenz, 2006; Viveros & Higgins, 2007; Dodson & Luer, 2010); La from *Lepanthopsis astrophora* a miniature epiphyte of coastal Colombia and Venezuela at 700 – 1500 m (Hokche *et al*., 2008; Idárraga-Piedrahita *et al*., 2011), and Mm from *Masdevallia menatoi*. Identification of the fungal strains using molecular techniques did not prove successful with isolates Df and Mm. Isolate La showed highest similarity to unidentified strains of root associating and mycorrhizal fungi from the fungal order Helotiales and class Leotiomycetes. Showing 98% identity to isolates of Leotiomycetes isolated from the lithophytic orchid: *Cattleya (Hoffmannseggella) cinnabarina* (Bateman ex Lindl.) Van den Berg from the Atlantic forests of Brazil (Oliveira *et al*., 2014). This isolate had a growth morphology similar to the Helotiale genera: *Phialocephala* and *Leptodontidium*, both of which are known mycorrhizal associates of orchids (Currah *et al*., 1987; 1988; Hou & Guo, 2009).

Experimental procedure

Seeds used in the study were mature and were obtained between the summer of 2015 and the spring of 2016. Flowers of *M. melanoxantha, M. menatoi, M. patchicutzae, R. contorta* were self-pollinated due to a lack of different individuals flowering at the same time. For *M. melanoxantha* there were sufficient individuals in flower to allow for cross pollination. This was done by removing the pollinia from the anther cap and then rubbing and placing them on the stigmatic surface of the column. In the case of *P. microptera, S. sp*. Peru and an example of *M. menatoi*, seed pods had formed spontaneously due to self-pollination in the greenhouse. For each species: seeds from different individuals and seed pods were combined, placed in greaseproof paper packets and dried in a plastic seed drying box with silica gel for 2 weeks at room temperature. Seed packets were then stored in an air-tight container with silica gel at 3±1 ºC until the seeds were sown in August 2016.

Fungi were isolated from orchid roots growing, in the case of *L. astrophora* and *M. menatoi* on cork bark pieces and for *D. felix* in a sphagnum moss and pine bark substrate. Roots were cut with a sterile scalpel that was wiped between cuttings with methylated spirits to prevent cross contamination. Roots were then rinsed and cut into 1 cm long pieces with a sterile scalpel following the same

procedure. The outer root surfaces were sterilised following a method described by Meissner (2014) for terrestrial orchids. For each species, root portions were placed in a beaker and soaked in 70% (v/v) ethanol for 1 minute, the beaker was then sprayed with 70% (v/v) ethanol and placed in a laminar flow cabinet (Flowfast V 15P, Faster D:Group, Ferrara, Italy) under aseptic conditions. Using sterile forceps, root portions were placed sequentially in beakers containing 30% (v/v) Hydrogen peroxide for 5 seconds, followed by transfer to 20% (v/v) Hydrogen peroxide for 20 seconds and then sterile deionised water for 30 seconds. Beakers were stirred throughout the process to ensure even treatment of root surfaces with the sterilant.

Root portions were then placed on sterile 90 mm petri dishes (Sterilin™, Thermo Fisher Scientific, Newport, UK) and cut into sub 0.5 mm sections using a sterile razor blade. Each section was then placed on 90 mm petri dish with 30ml of Potato Dextrose Agar (PDA), (Formedium, Hunstanton, UK). Petri dishes were subsequently sealed using Parafilm M® (Bemis, Neenah, WI, USA) and stored in a dark temperature controlled cabinet (LMS Series 4 Model 1200, LMS Ltd., Sevenoaks, Kent, UK) at 21±1 ºC. After treating the roots of each orchid species, all instruments were sterilised in 96% (v/v) ethanol before transfer to a glass bead steriliser (Keller Steri 350, Simon Keller AG, Burgdorf, Switzerland) at 250 ºC for 30 seconds. The surface of the laminar flow cabinet was wiped and sterilised with 70% (v/v) ethanol between sessions of working to prevent contamination. Cultures were assessed weekly for fungal growth. Where multiple fungi had grown from a root section, subcultures on PDA media were made under aseptic conditions to isolate the fungal strains. Fungi which had similar growth characteristics on PDA media to other symbiotic fungi utilised in Chapters 2, 3 with slower growth and lower hyphal densities were selected for symbiotic trials.

The following factors among treatments and within species were standardised: seed sterilisation technique; media volume; vessel size; temperature regime; light levels and photoperiod. Seeds of the studied species were sown on 55 mm petri dishes (Star™Dish, Phoenix Biomedical Products Inc., Bolton, Canada) containing 15 ml of media each. Symbiotic sowings were performed on modified H1 oat medium (Clements *et al*., 1986, modified by Rasmussen *et al*., 1990a). This media was chosen as it has been used extensively for the symbiotic culture of orchids (Clements *et al*., 1986; Muir, 1989;

Rasmussen *et al*., 1990a, 1990b; Wood & Ramsay, 2004; Zettler *et al*., 2007). Asymbiotic sowings were placed on full strength and half (1/2) strength Knudson C modified (KC) (Knudson, 1946), Sigma P6668 – Phytamax™ orchid maintenance medium (P6668) (Sigma-Aldritch Company Ltd., Gillingham, U.K.), Vacin and Went (VW) (Vacin & Went, 1949), modified with an equivalent iron molar concentration of ferrous sulfate in place of ferric tartrate (PhytoTechnology Laboratories, Shawnee Mission, U.S.A.), with all media prepared with 7 g l^{-1} of agar. In the case of *M. melanoxantha* additional treatments with 100 ml l^{-1} of coconut water (PhytoTechnology Laboratories, Shawnee Mission, U.S.A.) were added to all asymbiotic media at both half and full strength. These media were chosen as they have been widely and successfully used in the asymbiotic propagation of orchids (Knudson, 1946; Vacin & Went, 1949; Light & MacConaill, 2003; Lo *et al.*, 2004; Kauth *et al.*, 2005; 2006, 2008b; Stewart & Kane, 2006; Johnson *et al.*, 2007; Dutra *et al.*, 2008, 2009; Millner *et al.*, 2008; Millner, 2013).

Seed sterilisation was conducted under aseptic conditions in a laminar flow cabinet using the sterile syringe and mesh method (Ponert *et al*. 2011) with 48 micron nylon mesh (Plastok, Birkenhead, UK) and an initial 70% (v/v) ethanol treatment for 1 minute. Seeds of all species were then treated with 1% (w/v) NaOCl solution for 10 minutes. Sterilisation times and NaOCl solution strength were based on recommendations by Hicks (2000); McKendrick (2000), observation of seed bleaching from preliminary experiments with *M. menatoi* and *Stelis sp.* Peru and previous studies (Hughes, unpublished data; Chapter 3; 4). The preliminary experiment aimed to determine optimal sowing techniques. This involved sowings of mature seed of *M. menatoi* with the aforementioned procedure with the sterile syringe and mesh method. In the case of *Stelis sp.* Peru immature ‘green’ pods were sown prior to seed pod dehiscence. Seed pods were surface sterilised as per the method previously described for the surface sterilisation of orchid roots. Embryos were then excised from the seed pods using a sterile scalpel and spread over the media surface. Both species were sown on half strength P6668 media and cultured as detailed for the main experiment. Due to difficulties encountered with equally portioning immature embryos between treatments and treatment replicates, the sterile syringe and mesh method was chosen for this experiment.

The fungal isolate was inoculated onto symbiotic plates via the transfer of a 1 mm^3 piece of fungal culture containing fungal hyphae to the centre of each dish using sterile forceps. Upon completion of fungal inoculation for each orchid species, all instruments were sterilised in 96% (v/v) ethanol before transfer to a glass bead steriliser at 250 ºC for 30 seconds. The surface of the laminar flow cabinet was wiped and sterilised with 70% (v/v) ethanol between sessions of working to prevent contamination. Petri dishes were subsequently sealed using Parafilm M®. The number of different treatments tested were based on seed availability. These were as follows: For *M. melanoxantha*: ½ KC; ½ P6668; ½ VW; ½ KC+CW; ½ P6668+CW; ½ VW+CW; KC, P6668; VW; KC+CW; P6668+CW; VW+CW; H1; H1+Df; H1+La; H1+Mm. For *M. menatoi* and *P. microptera*: ½ KC; ½ P6668; ½ VW; KC; P6668; VW; H1; H1+Df; H1+La; H1+Mm. For *M. patchicutzae*: ½ KC; ½ P6668; ½ VW; H1+Mm. For *R. contorta*: ½ P6668; H1+Df. For *S. sp.* Peru: ½ KC; ½ P6668; ½ VW. Fifteen replicate plates were sown for each treatment giving a total of 240 plates for *M. melanoxantha*, 150 for *M. menatoi*, 60 for *M. patchicutzae*, 150 for *P. microptera*, 30 for *R. contorta* and 45 for *S. sp.* Peru. Seed number per plate (seed containing an embryo) varied as the sterilisation method did not allow for exact numbers of seed to be allocated to each plate, and species such as *R. contorta* had seed with low numbers of embryos. The average seed number per plate was: *M. melanoxantha*: 796.08 (SE = 34.1); *M. menatoi*: 245.87 (SE = 14.61); *M. patchicutzae*: 313.13 (SE = 26.95); *P. microptera*: 256.58 (SE = 22.96); *R. contorta*: 39.12 (SE = 6.66); *S. sp.* Peru: 540 (SE = 57.8).

All seed cultures were stored in a lit plant growth cabinet, Sanyo MLR-350 (Sanyo Electric Co. Ltd., Osaka Japan) at 21±1 ºC and a photoperiod of 12:12 h light/dark under Goodlight LED T8 20 W lights (LED Eco Lights Ltd., Camberley, U.K.) at 40 µmol m^{-2} s^{-1}. As preliminary experiments with *M. menatoi* and *Stelis sp.* Peru showed that embryos started to produce photosynthetic pigments within 1 – 2 weeks of sowing. Germination and development on petri-dishes was observed using a stereomicroscope (Leica M275, Leica Microsystems, Milton Keynes, UK) once every week for 10 weeks. Germination and development was recorded at 10 weeks and 20 weeks from the date of sowing. This was assessed by counting the number of seeds present with and without a visible embryo and noting the number and development stage of protocorms on each plate.

Protocorm development was scored using the method proposed by Clements *et al*. (1986): stage 1. germination of seed with rupture of testa; stage 2. production of rhizoids; stage 3. production of leaf primordium; stage 4. production of first chlorophyllous leaf tissue; stage 5. production of root initial, for figure see Chapter 3. This allowed for determination of mean percentage germination and development stage. Although this method was originally developed for terrestrial orchids the method generally still applied, though in some cases protocorms were observed to make a shoot primordium before any observable rhizoids. Where this occurred, the protocorms were classed as stage 3.

For each treatment, total numbers of germinants at specific development stages and overall germination were recorded for each plate. From this, percentages of germinants at each development stage and germination totals were calculated for each plate and means calculated for each treatment along with the standard deviation of the means. Statistical analysis was performed with germination data at 20 weeks of culture. Data was not analysed from week 10 due to minimal changes in germination between weeks 10 and 20. Analysis was conducted only on species with multiple treatments which had germination at 1% or higher. To analyse the effect of treatment, asymbiotic media strength, fungus (symbiotic vs asymbiotic), and the addition of coconut water to asymbiotic media on the proportion of germinates, a PERMANOVA analysis with 1000 permutations was performed. This method allowed us to analyse the data even if it was not normally distributed. These analyses were performed with the package “vegan” (Oksanen *et al*., 2017) in the R statistical environment (R core team, 2017).

Results

Final treatment replicate numbers for each species on each treatment were:

Masdevallia melanoxantha were: 15 for H1+Df; H1+Mm, 14 for H1+La, 13 for ½ VW; ½ KC+CW; VW+CW, 12 for P6668; VW, 11 for H1, 10 for ½ KC; ½ P6668; P6668+CW, 9 for ½ P6668+CW; KC+CW, 8 for KC, 4 for ½ VW+CW.

Masdevallia menatoi: 15 for KC; VW; H1+Df; H1+La; H1+Mm, 14 for ½ KC; ½ P6668, 13 for P6668; H1, 12 for ½ VW.

Masdevallia patchicutzae: 15 for ½ P6668; H1+Mm, 13 for ½ VW, 12 for ½ KC.

Pleurothallis microptera: 15 for ½ P6668; ½ VW; KC; P6668; VW; H1; H1+Df; H1+La; H1+Mm, 14 for ½ KC.

Restrepia contorta: 13 for H1+Df, 12 for ½ P6668.

Stelis sp. Peru: 14 for ½ KC, 11 for ½ VW, 10 for ½ P6668.

Replicate numbers per treatment were uneven due to the loss of some plates to contamination or lack of seed. Due to low germination (1% or lower) across treatments, statistical analysis was only performed on data from *M. melanoxantha*, *M. menatoi, P. microptera*.

Masdevallia melanoxantha

Germination

Masdevallia melanoxantha showed a highly significant effect of treatment upon germination at 20 weeks (F = 7.6924, df = 15, P = <0.001). Symbiotic germination at 10 weeks was highest on H1+Mm at 10 (52.1%±3.3) followed by H1+Df (17.9%±3.5) and was lowest on H1+La (1%±0.1) which was lower than the symbiotic H1 control (5.9%±0.7). Asymbiotic germination at 10 weeks was comparably lower, with the highest germination on P6668 (11.3%±1.1) and the lowest on KC+CW (3.8%±0.8), see Table 1.1, 2.1, Figure 1. The same trend was observed at 20 weeks with increases in germination in symbiotic H1+Mm (52.8%±3.6), H1+Df (18.5%±3.6), the H1 control (6.1%±0.7) and asymbiotic P6668 (11.8%±1) and KC+CW (3.9%±0.8), see Table 2.1, Figure 1. There was no effect of asymbiotic media strength (F = 2.2462, df = 1, p = 0.1618), though there was a highly significant effect of fungus (symbiotic vs asymbiotic treatments) with higher overall germination in symbiotic cultures (F = 24.538, df = 1, p = <0.001). There was no significant effect of the addition of coconut water to asymbiotic media (F = 0.4778, df = 1, p = 0.5055) on germination.

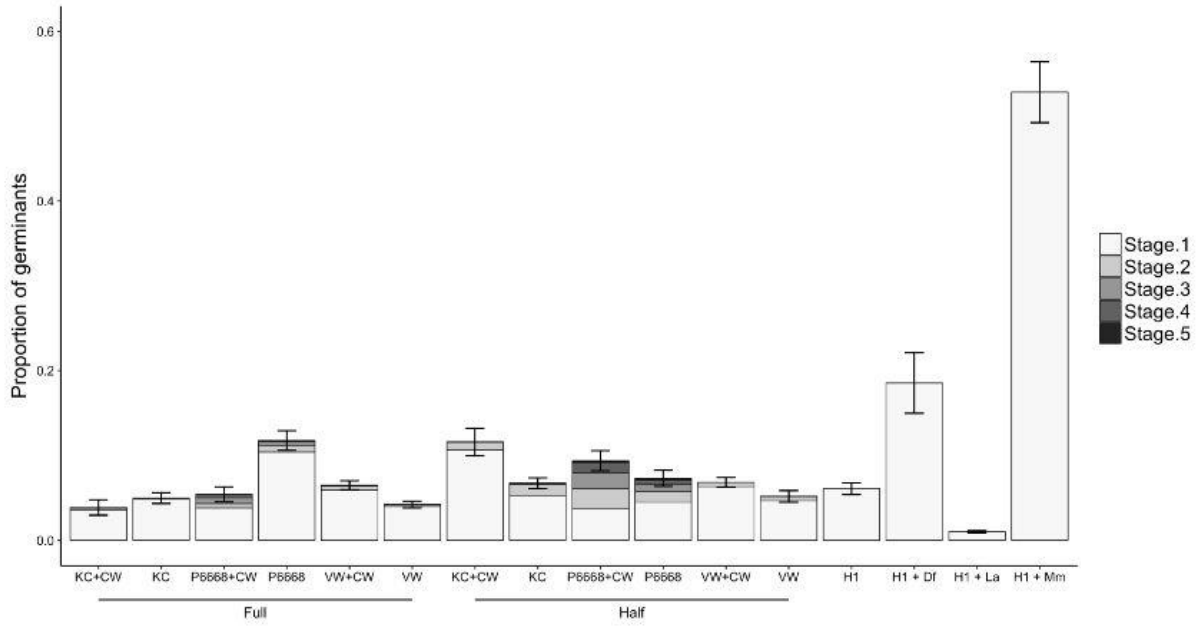

Figure 1. Germination and development of *Masdevallia melanoxantha* on half and full strength asymbiotic; Knudson C; Sigma P6668; Vacin and Went media with and without coconut water, H1 symbiotic media without fungus and symbiotic H1 media with fungal isolates Df (from *Dracula felix*), La (from *Lepanthopsis astrophora*) and Mm (from *Masdevallia menatoi*) at 20 weeks from sowing. Development stage 1 (white); stage 2 (light grey); stage 3 (grey); stage 4 (dark grey); stage 5 (black). Values represent the mean percentage of germinants at each development stage for each treatment. Error bars represent the standard error.

Development

After 20 weeks culture the maximum development stage observed in *M. melanoxantha* was stage 5 and was only observed on asymbiotic treatments ½ P6668 and ½ P6668+CW although this accounted for only 0.2%±0.1 of germination. Treatments: P6668+CW; P6668 developed as far as stage 4 with only 0.4%±0.2 and 0.2%±0.1 of germination at that stage respectively. Treatments: ½ KC; VW+CW; KC+CW developed as far as stage 3, but all with minimal germinants at this stage (0.1%±0.1). Treatments: ½ KC+CW; ½ VW; ½ VW+CW; VW; KC; H1+Df; H1+Mm developed as far as stage 2, for H1+Df and H1+Mm this consisted of only 1 germinant at stage 2. The symbiotic H1 control

and H1+La only reached stage 1. Although they didn't give the highest asymbiotic germination, the treatments: ½ P6668 (stage 3: 0.8%±0.1, stage 4: 0.5%±0.2, stage 5: 0.2%±0.1); P6668+CW (stage 3: 1.9%±0.2, stage 4: 1.2%±0.2, stage 5: 0.2%±0.1) and P6668+CW (stage 3: 0.7%±0.2, stage 4: 0.4%±0.2) had the highest proportion of germinants at advanced stages 3, 4 and 5. It should be noted the germinants with fungal strains Df and Mm were a brown colour in contrast to the clear and photosynthetic protocorm stages on asymbiotic treatments, (see Table 1.1 for week 10 and Table 2.1 for week 20 developmental data).

Masdevellia menatoi

Germination

Masdevallia menatoi showed a highly significant effect of treatment on germination ($F = 10.279$; $df = 9$; $p = <0.001$). Similar to *M. melanoxantha* at 10 weeks culture, symbiotic germination in *M. menatoi* was highest on H1+Mm (26.3%±3.3) followed by H1+Df (22.1%±3) and was lowest on H1+La (0.4%±0.1) which as in *M. melanoxantha* was lower than the symbiotic H1 control (5.3%±0.8). Asymbiotic germination at 10 weeks was comparably lower, with the highest germination on ½ P6668 (6.7%±0.6) and the lowest on ½ KC (0.2%±0.1), see Table 1.1. At 20 weeks, a similar trend was observed with increases in germination in H1+Mm (37.9%±4.9), H1+Df (37.5%±4.8), the symbiotic H1 control (5.4%±0.8) and asymbiotic ½ P6668 (7%±0.5), no further germination occurred on ½ KC, see Table 2.1, Figure 2. There was no significant effect of asymbiotic media strength ($F = 1.4266$; $df = 1$; $p = 0.2298$). A highly significant effect of fungus (symbiotic vs asymbiotic treatments) was observed with higher overall germination in symbiotic cultures ($F = 33.408$; $df = 1$; $p = <0.001$).

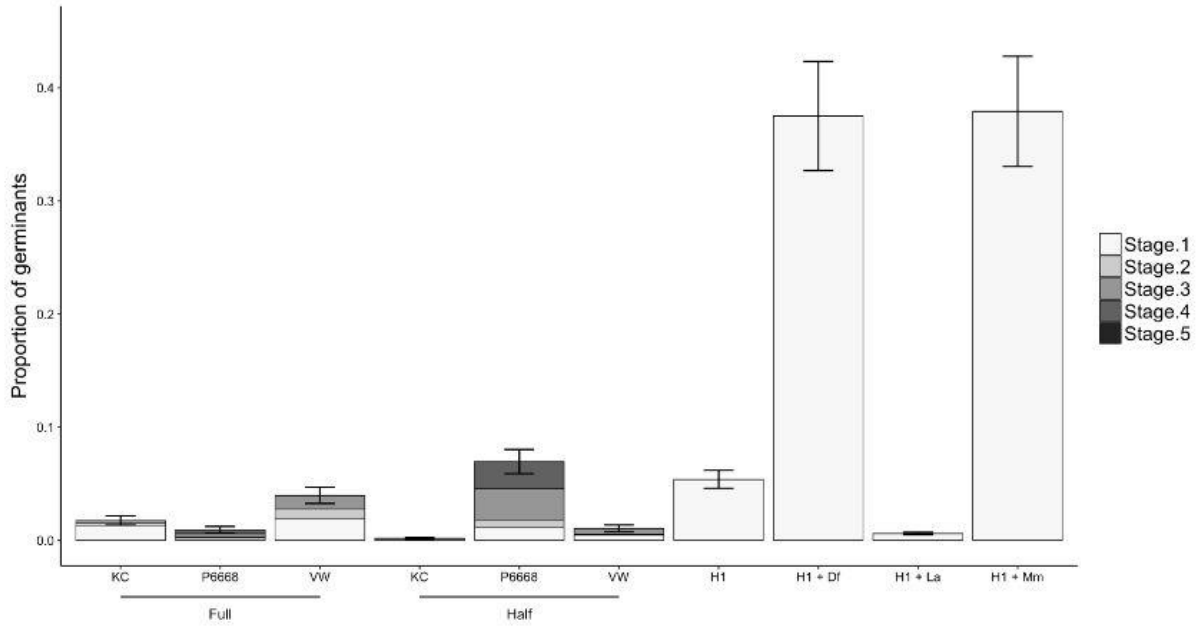

Figure 2. Germination and development of *Masdevallia menatoi* on half and full strength asymbiotic; Knudson C; Sigma P6668; Vacin and Went media, H1 symbiotic media without fungus and symbiotic H1 media with fungal isolates Df (from *Dracula felix*), La (from *Lepanthopsis astrophora*) and Mm (from *Masdevallia menatoi*) at 20 weeks from sowing. Development stage 1 (white); stage 2 (light grey); stage 3 (grey); stage 4 (dark grey); stage 5 (black). Values represent the mean percentage of germinants at each development stage for each treatment. Error bars represent the standard error.

Development

At 20 weeks culture the maximum development stage observed in *M. menatoi* was stage 4 and similarly to *M. melanoxantha,* furthest development was achieved with asymbiotic treatments. With ½ P6668 having the highest number of germinants at stage 4 (2.4%±0.3), followed by P6668 (0.3%±0.1). Treatments: ½ VW; KC; VW developed as far as stage 3. Despite having the highest germination H1+Mm and H1+Df only developed as far as stage 1, as did H1+La, ½ KC and the H1 control. Treatment: ½ P6668 (stage 3: 2.8%±0.3, stage 4: 2.4%±0.3) had the highest proportion of germinants at advanced stages 3 and 4 (Figure 3a). Note, germinants with fungal strains Df and Mm were a brown colour in contrast to the clear and photosynthetic protocorm stages on asymbiotic

treatments, (see Table 1.1 for week 10 and Table 2.1 for week 20 developmental data).

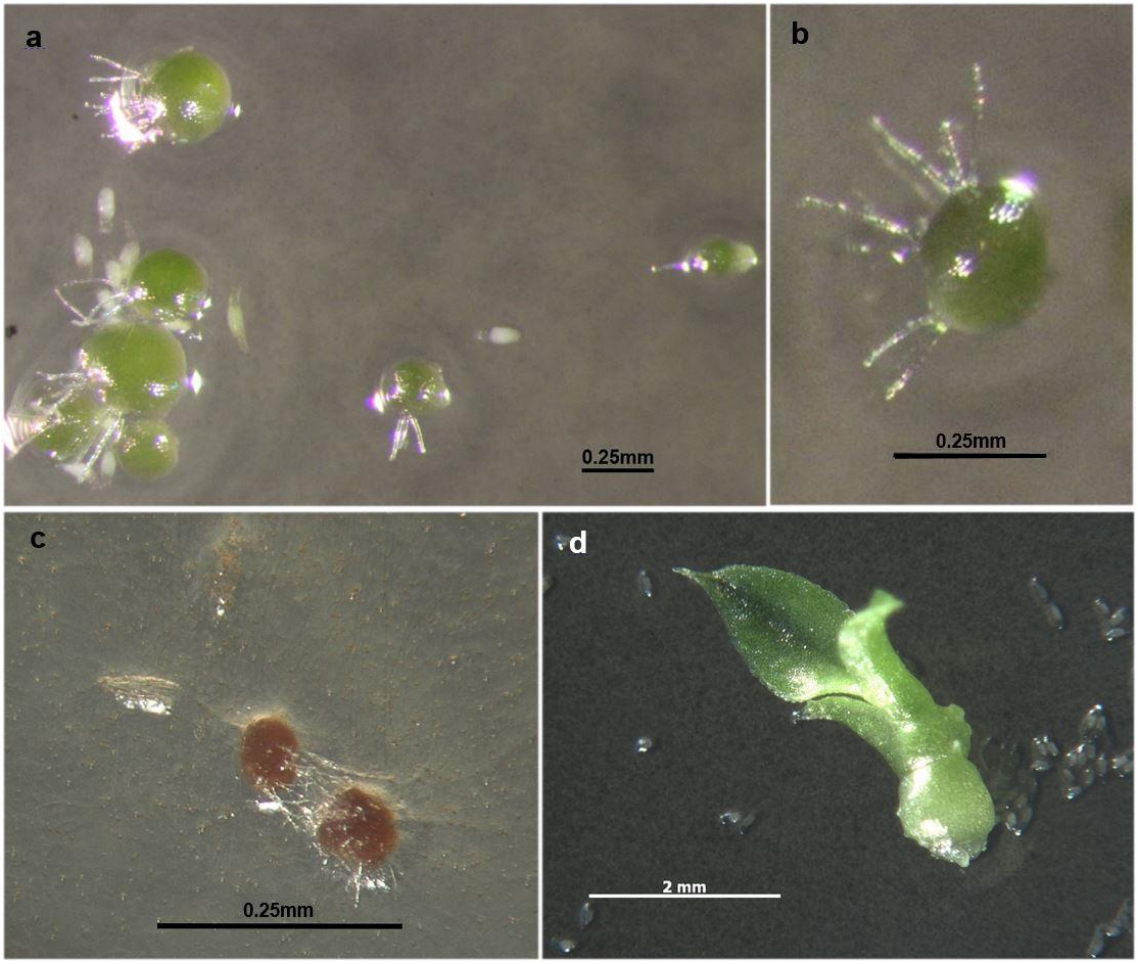

Figure 3. Stages of development: **a**) Ungerminated seed and stage 2 protocorms of *Masdevallia menatoi* displaying rhizoids on half strength P6668 asymbiotic media at 10 weeks from sowing (scale bar = 0.25 mm). **b**) Protocorm of *Stelis sp.* Peru at stage 2, displaying rhizoids on half strength P6668 asymbiotic media at 10 weeks from sowing (scale bar = 0.25 mm). **c**) Brown coloured protocorms of *Restrepia contorta* at stage 1 with Df (*Dracula felix*) fungal isolate at 20 weeks from sowing, note: to left of the two protocorms is an unviable seed without an embryo (scale bar = 0.25 mm). **d**) *Pleurothallopsis microptera* germinant at stage 5 with first leaves and formation of root initials, amongst ungerminated seed on half strength P6668 asymbiotic media at 20 weeks from sowing (scale bar = 2 mm).

Masdevallia patchicutzae

Germination

No statistical analyses could be undertaken on this data due to low asymbiotic germination. At 10 weeks culture, germination in *M. patchicutzae* was highest with the symbiotic, H1+Mm (22.6%±1.8). Asymbiotic germination was comparatively very low, with the highest germination on ½ KC (0.8%±0.3) followed by ½ P6668 (0.3%±0.1), ½ VW (0.2%±0.1), see Table 1.2. At 20 weeks culture germination increased with the same trend H1+Mm (40.3%±4) followed by ½ KC (1%±0.4), ½ P6668 (0.4%±0.1) with exception to ½ VW, where no further germination occurred, see Table 2.2.

Development

After 20 weeks culture the maximum development stage was observed in *M. patchicutzae* was stage 4 on ½ P6668 (stage 1: 0.1%±0, stage 2: 0, stage 3: 0.2%±0.1, stage 4: 0.1%±0.1). Treatment ½ KC developed as far as stage 3, with a higher proportion of germinanats at earlier stages 1 and 2 (stage 1: 0.5%±0.2, stage 2: 0.5%±0.3, stage 3: 0.1%±0). Similarly to *M. melanoxantha* and *M. menatoi*, despite having the highest germination on H1+Mm, germinants only developed as far as stage 1 at 20 weeks culture, as did asymbiotic ½ VW. Note, germinants with fungal strain Mm were a brown colour in contrast to the clear and photosynthetic protocorm stages on asymbiotic treatments, (see Table 1.2 for week 10 and Table 2.2 for week 20 developmental data).

Pleurothallopsis microptera

Germination

Pleurothallopsis microptera showed a significant effect of treatment on germination ($F = 8.785$; $df = 6$; $p = <0.001$). After 10 weeks culture, symbiotic germination in *P. microptera* was highest on H1+Mm (16.2%±4.1), followed by H1+Df (9.1%±2.4). Asymbiotic germination was highest on ½ P6668 (4.7%±2.6) with P6668, VW, ½ KC, ½ VW giving very low germination (<0.2%). No germination was observed on treatments KC, H1, H1+La, see Table 1.2. At 20 weeks culture, an increase in germination was only observed in H1+Mm (31.1%±5.5), H1+Df (14.1%±3.4), ½ P6668 (4.8%±2.6). Treatments P6668, VW,

½ KC and ½ VW did not induce any further germination and as at week 10, no germination was observed on treatments KC, H1 and H1+La (see Table 2.2, Figure 4). There was no significant effect of asymbiotic media strength (F = 2.1774; df = 1; p = 0.1688). A highly significant effect of fungus (symbiotic vs asymbiotic treatments) was observed with higher overall germination in symbiotic cultures (F = 41.883; df = 1; p = <0.001).

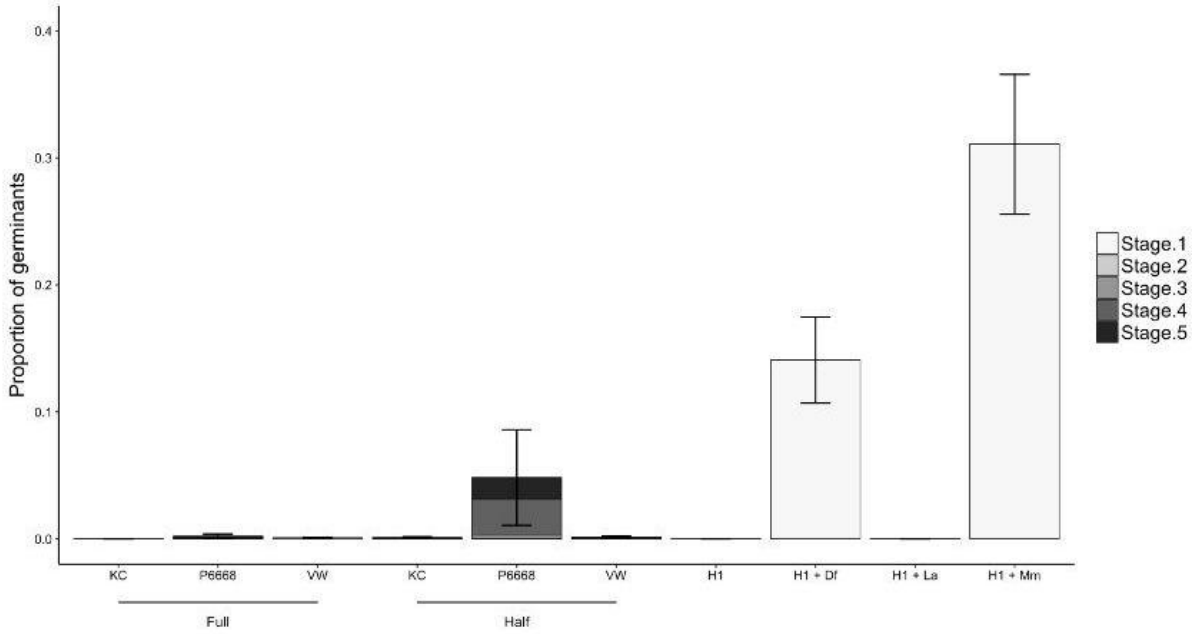

Figure 4. Germination and development of *Pleurothallopsis microptera* on half strength asymbiotic Knudson C; Sigma P6668; Vacin and Went media and full strength asymbiotic; Sigma P6668; Vacin and Went media, symbiotic H1 media with fungal isolates Df (from *Dracula felix*), and Mm (from *Masdevallia menatoi*) at 20 weeks from sowing. Development stage 1 (white); stage 2 (light grey); stage 3 (grey); stage 4 (dark grey); stage 5 (black). Values represent the mean percentage of germinants at each development stage for each treatment. Error bars represent the standard error.

Development

Similar to the previous species, at 20 weeks culture the maximum development stage observed in *P. microptera* was stage 5 with treatments: ½ P6668 most successful, producing the highest proportion of germinants at advanced stages 3, 4 and 5 (stage 3: 0.3%±0.3, stage 4: 2.8%±2.3, stage 5: 1.7%±1.2), (Figure

3d). This was followed by P6668 (stage 3: 0.1%±0.1, stage 4: 0, stage 5: 0.1%±0.1). Treatment ½ VW had very low germination with all germinants developed as far as stage 3, with a very small proportion at this stage (0.1%±0.05). As observed in *M. menatoi*, *M. patchicutzae*, *P. microptera* and similarly *M. melanoxantha*, symbiotic treatments H1+Mm and H1+Df only developed as far as stage 1, as did asymbiotic ½ KC and VW. Note, germinants with fungal strain Mm were a brown colour in contrast to the clear and photosynthetic protocorm stages on asymbiotic treatments, (see Table 1.2 for week 10 and Table 2.2 for week 20 developmental data).

Restrepia contorta

Germination and development

No statistical analyses could be undertaken on this data due to low asymbiotic germination. At 10 weeks culture, germination in *R. contorta* was highest on H1+Df (15%±4.2) at stage 1 compared to ½ P6668 (0.1%±0.1), see Table 1.2. It should be noted that germination on ½ P6668 represented a single germinant at stage 2 and observations of seed post sowing revealed that a large number of seed did not have embryos. At 20 weeks culture germination increased in treatment H1+Df (20.2%±4.2) with germinants not progressing beyond stage 2. Germination did not increase for treatment ½ P6668, nor had the single germinant developed beyond stage 2, see Table 2.2. As with the other species, germinants on fungal strain Df were brown in colour (Figure 3c).

Stelis sp. Peru

Germination and development

No statistical analyses could be undertaken on this data due to low germination. At 10 weeks culture, germination in *S. sp.* Peru was fairly low and with the highest germination on ½ P6668 (1.1%±0.4), followed by ½ KC (0.6%±0.2) and ½ VW (0.5%±0.1), see Table 1.2. At 20 weeks culture germination had only increased with ½ P6668 (1.2%±0.4) which also had the highest development at stage 3 and 4: ½ P6668 (stage 3: 0.8%±0.3, stage 4: 0.1%±0.1), (Figure 3b). Treatment ½ VW developed as far as stage 2 (stage 1: 0.3%±0.1, stage 2: 0.2%±0) and ½ KC only to stage 1, see Table 1.2, 2.2.

Table 1.1. Protocorm development and germination of *Masdevallia melanoxantha; Masdevallia menatoi* at 10 weeks from sowing on half and full strength asymbiotic; Knudson C; Sigma P6668; Vacin and Went media with and without coconut water, H1 symbiotic media without fungus and symbiotic H1 media with fungal isolates Df (from *Dracula felix*), La (from *Lepanthopsis astrophora*) and Mm (from *Masdevallia menatoi*). Numbers in parenthesis represent standard error of the data.

Species	Treatment	Mean % in developmental stage					Mean % Germination
		Stage 1	Stage 2	Stage 3	Stage 4	Stage 5	Overall
Masdevallia melanoxantha	1/2 KC	6.4 (0.5)	0.3 (0.1)	0 (0)	0 (0)	0 (0)	6.7 (0.5)
	1/2 KC + CW	10.7 (1.2)	0 (0)	0 (0)	0 (0)	0 (0)	10.7 (1.2)
	1/2 P6668	5.5 (0.6)	1.5 (0.2)	0.1 (0.03)	0 (0)	0 (0)	7.1 (0.6)
	1/2 P6668 + CW	6.2 (0.6)	2.7 (0.4)	0.2 (0.1)	0 (0)	0 (0)	9.1 (0.8)
	1/2 VW	4.6 (0.6)	0.1 (0.1)	0 (0)	0 (0)	0 (0)	4.8 (0.6)
	1/2 VW + CW	6.6 (0.5)	0.2 (0.1)	0 (0)	0 (0)	0 (0)	6.8 (0.6)
	KC	4.9 (0.6)	0.1 (0.04)	0 (0)	0 (0)	0 (0)	5 (0.6)
	KC + CW	3.8 (0.8)	0.1 (0.03)	0 (0)	0 (0)	0 (0)	3.8 (0.8)
	P6668	10.2 (0.8)	1 (0.3)	0.1 (0.04)	0 (0)	0 (0)	11.3 (1.1)
	P6668 + CW	4.3 (0.4)	1 (0.2)	0.2 (0.1)	0 (0)	0 (0)	5.4 (0.6)
	VW	4.1 (0.4)	0.1 (0.02)	0 (0)	0 (0)	0 (0)	4.2 (0.4)
	VW + CW	5.8 (0.4)	0.1 (0.03)	0 (0)	0 (0)	0 (0)	5.8 (0.4)
	H1	5.9 (0.7)	0 (0)	0 (0)	0 (0)	0 (0)	5.9 (0.7)
	H1 + Df	17.8 (3.5)	0.01 (0.01)	0	0	0	17.9 (3.5)
	H1 + La	1 (0.1)	0	0	0	0	1 (0.1)
	H1 + Mm	52.1 (3.3)	0	0	0	0	52.1 (3.3)
Masdevallia menatoi	1/2 KC	0.2 (0.1)	0 (0)	0 (0)	0 (0)	0 (0)	0.2 (0.1)
	1/2 P6668	2.3 (0.5)	4.4 (0.3)	0 (0)	0 (0)	0 (0)	6.7 (0.6)
	1/2 VW	0.9 (0.2)	0 (0)	0 (0)	0 (0)	0 (0)	0.9 (0.2)
	KC	1.7 (0.3)	0.1 (0.06)	0 (0)	0 (0)	0 (0)	1.7 (0.3)
	P6668	0.4 (0.1)	0.5 (0.1)	0 (0)	0 (0)	0 (0)	0.9 (0.2)
	VW	3.5 (0.3)	0.1 (0.1)	0 (0)	0 (0)	0 (0)	3.6 (0.3)
	H1	5.3 (0.8)	0 (0)	0 (0)	0 (0)	0 (0)	5.3 (0.8)
	H1 + Df	22.1 (3)	0	0	0	0	22.1 (3)
	H1 + La	0.4 (0.1)	0	0	0	0	0.4 (0.1)
	H1 + Mm	26.3 (3.3)	0	0	0	0	26.3 (3.3)

Table 1.2. Protocorm development and germination of *Masdevallia patchicutzae; Pleurothallopsis microptera; Restrepia contorta; Stelis sp.* Peru at 10 weeks from sowing on half and full strength asymbiotic; Knudson C; Sigma P6668; Vacin and Went media, H1 symbiotic media without fungus and symbiotic H1 media with fungal isolates Df (from *Dracula felix*), La (from *Lepanthopsis astrophora*) and Mm (from *Masdevallia menatoi*). Numbers in parenthesis represent standard error of the data. * This treatment had only one germinant.

Species	Treatment	Mean % in developmental stage					Mean % Germination
		Stage 1	Stage 2	Stage 3	Stage 4	Stage 5	Overall
Masdevallia patchicutzae	1/2 KC	0.7 (0.3)	0.1 (0.1)	0 (0)	0 (0)	0 (0)	0.8 (0.3)
	1/2 P6668	0.06 (0.04)	0.26 (0.07)	0 (0)	0 (0)	0 (0)	0.3 (0.1)
	1/2 VW	0.2 (0.1)	0.03 (0.03)	0 (0)	0 (0)	0 (0)	0.2 (0.1)
	H1 + Mm	22.6 (1.8)	0	0	0	0	22.6 (1.8)
Pleurothallopsis microptera	1/2 KC	0.1 (0.04)	0 (0)	0 (0)	0 (0)	0 (0)	0.1 (0.04)
	1/2 P6668	0.7 (0.6)	0 (0)	3.9 (2.4)	0 (0)	0 (0)	4.7 (2.6)
	1/2 VW	0.1 (0.05)	0 (0)	0 (0)	0 (0)	0 (0)	0.1 (0.05)
	KC	0	0	0	0	0	0
	P6668	0 (0)	0 (0)	0.2 (0.1)	0 (0)	0 (0)	0.2 (0.1)
	VW	0.1 (0.03)	0	0	0	0	0.1 (0.03)
	H1	0	0	0	0	0	0
	H1 + Df	9.1 (2.4)	0	0	0	0	9.1 (2.4)
	H1 + La	0	0	0	0	0	0
	H1 + Mm	16.2 (4.1)	0	0	0	0	16.2 (4.1)
Restrepia contorta	1/2 P6668*	0	0.1 (0.1)	0	0	0	0.1 (0.1)
	H1 + Df	15 (4.2)	0	0	0	0	15 (4.2)
Stelis sp. Peru	1/2 KC	0.6 (0.2)	0	0	0	0	0.6 (0.2)
	1/2 P6668	0.4 (0.2)	0.6 (0.4)	0.03 (0.03)	0 (0)	0 (0)	1.1 (0.4)
	1/2 VW	0.5 (0.1)	0.01 (0.01)	0 (0)	0 (0)	0 (0)	0.5 (0.1)

Table 2.1. Protocorm development and germination of *Masdevallia melanoxantha; Masdevallia menatoi* at 20 weeks from sowing on half and full strength asymbiotic; Knudson C; Sigma P6668; Vacin and Went media with and without coconut water, H1 symbiotic media without fungus and symbiotic H1 media with fungal isolates Df (from *Dracula felix*), La (from *Lepanthopsis astrophora*) and Mm (from *Masdevallia menatoi*). Numbers in parenthesis represent standard error of the data.

Species	Treatment	Mean % in developmental stage					Mean % Germination
		Stage 1	Stage 2	Stage 3	Stage 4	Stage 5	Overall
Masdevallia melanoxantha	1/2 KC	5.3 (0.4)	1.4 (0.2)	0.1 (0)	0	0	6.7 (0.4)
	1/2 KC + CW	10.7 (1.4)	0.9 (0.2)	0	0	0	11.6 (1.5)
	1/2 P6668	4.5 (0.5)	1.3 (0.2)	0.8 (0.1)	0.5 (0.2)	0.2 (0.1)	7.3 (0.6)
	1/2 P6668 + CW	3.7 (0.5)	2.4 (0.2)	1.9 (0.2)	1.2 (0.2)	0.2 (0.1)	9.4 (0.9)
	1/2 VW	4.7 (0.6)	0.5 (0.1)	0	0	0	5.2 (0.6)
	1/2 VW + CW	6.3 (0.5)	0.5 (0.1)	0	0	0	6.8 (0.6)
	KC	4.9 (0.6)	0.1 (0)	0	0	0	5 (0.6)
	KC + CW	3.6 (0.8)	0.2 (0.1)	0.1 (0.1)	0	0	3.9 (0.8)
	P6668	10.4 (0.8)	0.7 (0.1)	0.5 (0.1)	0.2 (0.1)	0	11.8 (1)
	P6668 + CW	3.8 (0.4)	0.5 (0.2)	0.7 (0.2)	0.4 (0.2)	0	5.4 (0.6)
	VW	4 (0.3)	0.2 (0.1)	0	0	0	4.2 (0.4)
	VW + CW	5.9 (0.4)	0.5 (0.1)	0.1 (0)	0	0	6.5 (0.5)
	H1	6.1 (0.7)	0	0	0	0	6.1 (0.7)
	H1 + Df	18.5 (3.6)	0.01 (0.01)	0	0	0	18.5 (3.6)
	H1 + La	1 (0.1)	0	0	0	0	1 (0.1)
	H1 + Mm	52.8 (3.6)	0	0	0	0	52.8 (3.6)
Masdevallia menatoi	1/2 KC	0.2 (0.1)	0	0	0	0	0.2 (0.1)
	1/2 P6668	1.1 (0.3)	0.6 (0.2)	2.8 (0.3)	2.4 (0.3)	0	7 (0.5)
	1/2 VW	0.5 (0.1)	0.1 (0)	0.5 (0.2)	0	0	1 (0.2)
	KC	1.3 (0.2)	0.2 (0.1)	0.2 (0.1)	0	0	1.7 (0.3)
	P6668	0.2 (0.1)	0	0.3 (0.1)	0.3 (0.1)	0	0.9 (0.2)
	VW	1.9 (0.3)	0.9 (0.2)	1.2 (0.2)	0	0	4 (0.4)
	H1	5.4 (0.8)	0	0	0	0	5.4 (0.8)
	H1 + Df	37.5 (4.8)	0	0	0	0	37.5 (4.8)
	H1 + La	0.6 (0.1)	0	0	0	0	0.6 (0.1)
	H1 + Mm	37.9 (4.9)	0	0	0	0	37.9 (4.9)

Table 2.2. Protocorm development and germination of *Masdevallia patchicutzae; Pleurothallopsis microptera; Restrepia contorta; Stelis sp.* Peru at 20 weeks from sowing on half and full strength asymbiotic; Knudson C; Sigma P6668; Vacin and Went media, H1 symbiotic media without fungus and symbiotic H1 media with fungal isolates Df (from *Dracula felix*), La (from *Lepanthopsis astrophora*) and Mm (from *Masdevallia menatoi*). Numbers in parenthesis represent standard error of the data. * This treatment had only one germinant.

Species	Treatment	Mean % in developmental stage					Mean % Germination
		Stage 1	Stage 2	Stage 3	Stage 4	Stage 5	Overall
Masdevallia patchicutzae	1/2 KC	0.5 (0.2)	0.5 (0.3)	0.1 (0)	0	0	1 (0.4)
	1/2 P6668	0.1 (0)	0	0.2 (0.1)	0.1 (0.1)	0	0.4 (0.1)
	1/2 VW	0.2 (0.1)	0	0	0	0	0.2 (0.1)
	H1 + Mm	40.3 (4)	0	0	0	0	40.3 (4)
Pleurothallopsis microptera	1/2 KC	0.1 (0.04)	0	0	0	0	0.1 (0.04)
	1/2 P6668	0	0	0.3 (0.3)	2.8 (2.3)	1.7 (1.2)	4.8 (2.6)
	1/2 VW	0	0	0.1 (0.05)	0	0	0.1 (0.05)
	KC	0	0	0	0	0	0
	P6668	0	0	0.1 (0.1)	0	0.1 (0.1)	0.2 (0.1)
	VW	0.1 (0.03)	0	0	0	0	0.1 (0.03)
	H1	0	0	0	0	0	0
	H1 + Df	14.1 (3.4)	0	0	0	0	14.1 (3.4)
	H1 + La	0	0	0	0	0	0
	H1 + Mm	31.1 (5.5)	0	0	0	0	31.1 (5.5)
Restrepia contorta	1/2 P6668*	0	0.1 (0.1)	0	0	0	0.1 (0.1)
	H1 + Df	20.2 (4.2)	0	0	0	0	20.2 (4.2)
Stelis sp. Peru	1/2 KC	0.6 (0.2)	0	0	0	0	0.6 (0.2)
	1/2 P6668	0	0.2 (0.1)	0.8 (0.3)	0.1 (0.1)	0	1.2 (0.4)
	1/2 VW	0.3 (0.1)	0.2 (0)	0	0	0	0.5 (0.1)

Discussion

The hypothesis that lower asymbiotic media concentrations would have a positive effect on germination proved to be incorrect in *M. melanoxantha, M. menatoi* and *P. microptera*. As there was no significant difference between asymbiotic media strengths. The hypothesis that the presence of coconut water in asymbiotic media would have a positive effect on germination and development in *M. melanoxantha* also proved to be incorrect, with no significant effect detected by its addition. The hypothesis that symbiotic fungi would induce different responses for each orchid species to each tested fungus did prove to be partially correct. Germination was highest with the fungus Mm, but furthest development was supported on asymbiotic ½ P6668 media.

A highly significant effect of fungus was found in the germination of *M. melanoxantha*, *M. menatoi*, *P. microptera.* Fungal strains Mm followed by Df proved to be the best germinators, giving germination values (14 – 53%) several times higher than respective asymbiotic treatments (<12%) in *M. melanoxantha, M. menatoi*, *M. patchicutzae*, *P. microptera, R. contorta*. The symbiotic advantage in germination corresponds with similar results obtained in other epiphytic (Otero and Bayman, 2009) and terrestrial orchids (Rasmussen *et al*., 1990a; Anderson, 1991; Chapter 3; 4; 5). Germination responses to fungal strains Mm and Df differed between species, with both *M. melanoxantha* and *P. microptera* having more than twice as much germination with Mm. *Masdevallia menatoi* in contrast had similar germination with Mm and Df. These differing species specific responses to fungi upon germination have also been observed in epiphytic (Otero & Bayman, 2009; Nontachaiyapoom *et al.,* 2011: Hajong *et al*., 2013) and terrestrial orchids (Warcup, 1973; 1981; 1988; Muir, 1989; Chapter 3; 4; 5).

The fungal strain La induced minimal germination and development in *M. melanoxantha* and *M. menatoi* and in the case of *P. microptera* no germination at all. Considering that germination was higher in all cases with the H1 control, it is likely this fungus was inhibiting germination in these species. Symbiotic controls with seeds sown solely on H1 media did result in germination, although not in *P. microptera,* with germinants of *M. melanoxantha*, *M. menatoi* only developing as far as stage 1. In both cases, germination with Mm and Df fungal treatments was several times higher than the H1 control, indicating that these

results were due to fungal interaction. The fungal treatments Mm and Df resulted in the formation of brown protocorms, mostly at stage 1, with the maximum development, stage 2, being observed only in single protocorms of *M. melanoxantha* with the fungi Mm and Df. The brown colouring of the protocorms may be a result of the brown hyphal coils present in the protocorm cells (Warcup, 1990) or due to parasitism by the fungus (Hadley, 1969). Further culture may help determine which of these is the case. If these results are compared with favourable orchid-fungal pairings as seen in Chapters: 2; 3; 4; 5, it is evident that these fungi did not possess the potential to be optimal symbionts *in vitro*.

In *R. contorta* a limited amount of seed only allowed for one asymbtotic and one symbiotic treatment and a large number of those seeds did not have embryos. Despite this there was an average of 39.12 (SE = 6.66) seeds with embryos per plate. The fungal strain Df resulted in germination comparable to other species but the asymbiotic ½ P6668 media produced only one germinant. This asymbiotic media at full strength has shown to be effective at germinating; mean germination 26.66% and producing viable seedlings of *Restrepia brachypus* Rchb.f (Millner *et al*., 2008). The poor germination results may have been due to artificially self-pollinating the parent plants (Millner, 2013; Millner *et al*., 2015).

Self-incompatibility has been demonstrated in species from the Pleurothallid genera: *Acianthera* (Borba *et al*., 2001; 2011); *Anathallis* (Gontijo *et al*., 2010; Borba *et al*., 2011); *Lepanthes* (Tremblay *et al*., 2005); *Octomeria* (Barbosa *et al*., 2009; Borba *et al*., 2011); *Restrepia* (Millner *et al*., 2015); *Specklinia*; (Borba *et al*., 2011); *Stelis* (Christensen, 1992; Borba *et al*., 2011). There were numerous failed attempts at artificial pollination with many genera, including: *Acianthera, Anathallis, Dracula, Dryadella, Lepanthopsis, Masdevallia, Pleurothallis, Restrepia, Specklinia, Stelis spp.* in the national plant collection, most of which were self-pollinated due to a lack of other flowering individuals. Whilst this may also be due to poor technique and difficulties associated with flower morphology, self-incompatibility is also a likely cause. My own observations of ‘spontaneous’ self-pollinated seed pods of various species within the subtribe in the collections greenhouses, showed that most had none or very little viable seed. There were some notable exceptions: *P. microptera, Stelis sp*. Peru and an example of *M. menatoi* that were used in this study. Borba *et al*.,

(2011) have also shown that some plants within populations of *Masdevallia infracta* Lindl. can exhibit self-incompatibility whilst others are self-compatible. There are also some species within the genera: *Acianthera*, *Octomeria* (Borba *et al*., 2011) which demonstrated partial self-incompatibility and others which are self-compatible: *Acianthera aphthosa* (Lindl.) Pridgeon & M.W.Chase (Pansarin *et al*., 2016); *Zootrophion atropurpureum* Lindl. (Borba *et al*., 2011). The results of this and the aforementioned studies show that self-incompatibility is an important factor when considering the *ex situ* conservation of the subtribe. Plants of subtribe Pleurothallidinae are often propagated *ex situ* via asexual division of plants or adventitious growths, known as 'keiki's' (Millner *et al*., 2008). It is vital that where e*x situ* populations exist that multiple genotypes of each species are maintained, ensuring the possibility of sexual reproduction and maintenance of genetic diversity.

When the orchid species were compared individually there was some individual preferences for different asymbiotic media treatments, more so than that observed with fungal treatments. *Masdevallia melanoxantha* had the highest germination on P6668, ½ KC+CW and ½ P6668+CW but the most advanced development occurred on treatments: ½ P6668+CW; ½ P6668; P6668+CW. The results for *M. melanoxantha* did not prove conclusive in determining an optimal asymbiotic media. In terms of the highest proportions of advanced seedlings at 20 weeks culture; ½ P6668+CW would appear to be the most effective. However, with more time in culture treatments P6668, ½ P6668 may prove equally effective. This would negate the need for adding additional complex organic media components and make for more efficient propagation. Asymbiotic germination and development in *M. menatoi* and *P. microptera* at 20 weeks was highest with ½ P6668 and to a lesser extent full strength P6668. *Masdevallia patchicutzae* and *Stelis sp.* Peru germinated very poorly on all three half strength asymbiotic media tested <1%. In *M. patchicutzae*, ½ KC gave the best germination whereas *Stelis sp.* Peru germinated best on ½ P6668. Germinants in both species, however, developed furthest on ½ P6668. To my knowledge there are no studies on germination of Pleurothallid orchids utilising half strength P6668 media. Millner *et al*., (2008) have shown full strength P6668 to be effective in germination and development of *R. brachypus*. The highest germination in *R. brachypus*, however, occurred with full strength Western media, with optimal development

obtained when 60 g l^{-1} of banana pulp was added to this media. The statement that different *Masdevallia* species display different preferences to media strength and composition upon germination (Mckendrick, 2000) proved to be generally correct in this study. However, on the whole, ½ P6668 proved to be the optimal asymbiotic media for production of viable seedlings, though further experimentation is necessary to improve germination percentages.

The lower germination observed on asymbiotic cultures could be due to sub-optimal seed scarification and/or asymbiotic media formulation, seed age or another factor. The preliminary sowing using mature seed of *M. menatoi* did result in far higher germination. Though germination was not quantified as in this study, a similar amount of seed was used that would be equivalent to one of the 15 replicate treatments in this study. After 2 to 3 year's culture more than 800 plantlets were de-flasked, and at 3 years culture the most advanced seedlings were of a comparable size to adult plants. The ½ P6668 treatment for this species in this study resulted in 188 germinants at 20 weeks which was considerably less than the preliminary experiment. The only notable difference between the two experiments was the period of seed storage which was around 2 weeks in the preliminary study compared to 3 months in this study. This could suggest that seed longevity may be short in *M. menatoi* or that the seed storage method was not optimal. Further investigation is necessary to determine the longevity of Pleurothallid seed.

Clavijo, (2010) investigated sowing seed from immature, indehiscent seed capsules of *Masdevallia tovarensis* on variations of Murashige and Skoog media modified with different phytohormones. Though germination did not differ greatly from that observed with mature seed of *Masdevallia* species in this study. A preliminary study sowing three immature, indehiscent seed capsules of *S. sp.* Peru on ½ P6668 gave somewhat better results than those noted here. Unfortunately, germination was not quantified as in this study, but more than 700 seedlings were de-flasked after 3 years culture. Compared to the preliminary study, germination in this experiment with mature seed was far less favourable with only 22 germinants out of 3715 seeds on the same treatment at 20 weeks. This may indicate that there is an inbuilt factor in mature seed preventing germination. Seeds could also have been sensitive to the sterilant used or received excessive bleaching. Millner *et al*., (2008) treated seed of *R. brachypus*

for 10 minutes using a weaker solution (0.5% w/v) NaOCl, compared to the 1% (w/v) NaOCl used for the same time period in this study. Previous attempts with sowing epiphytic orchids using 0.5% (w/v) NaOCl had resulted in an excessive amount of culture contamination and was not used further (Hughes, unpublished data). Mature seeds in this case were stored for three to six months which as with *M. menatoi* may have been a factor which resulted in poor germination.

When using asymbiotic methods for conserving orchids, it is vital to conserve genetic diversity for maintenance of healthy populations. Using immature, indehiscent seed capsules can result in lack of genetic diversity in germinants due to the potential culture of maternal capsule material which can produce clonal protocorms (Zettler *et al*., 2007). Additionally, this method does not prevent the spread of viral infections from parent plants (Ramsay & Dixon, 2003) which are often absent in wild populations (Zettler *et al*., 2007). Due to these flaws and difficulties encountered with equally portioning immature embryos between treatments and treatment replicates, further investigation was not conducted.

Asymbiotic methods could reliably produce viable plantlets *in vitro*, but some studies have experienced problems with seedling establishment *ex vitro* (Stenberg & Kane, 1998). Zettler *et al*., (2007), demonstrated that symbiotic fungi can be used to produce epiphytic orchid *Epidendrum nocturnum* Jacq. *in vitro* and successfully reintroduce them to suitable habitats. This study utilised a fungus isolated from a terrestrial orchid *Spiranthes brevilabris* Lindl. from the region. This shows that suitable symbionts may be found in distant taxa, isolating fungi from a broader range of orchids may help find suitable symbionts. Though this should be focused on orchids within the same regions and habitats so that fungi are not introduced into alien environs when symbiotic seedlings are reintroduced.

The fungal strains Df and Mm proved to be the most effective fungal symbionts and gave far higher germination than any of the asymbiotic treatments. Unfortunately, these fungi did not result in advanced seedling development with any of the species tested. Symbiotic orchid seedlings have been shown to have higher survival rates *ex vitro* (Anderson, 1991; Ramsay & Dixon, 2003; Aggarwal & Zettler, 2010; Chapter 4). This is due to the benefits of mycorrhizal fungi;

improving nutrient exchange, and nutrient (Cameron *et al*., 2006; 2007) and water mobilisation (Yoder *et al*., 2000). As well as potentially protecting seedlings from other microorganisms. Utilisation of symbiotic methods can also be used to improve degraded habitats by introducing appropriate mycorrhizal fungi, helping to ensure future orchid recruitment (Zettler, 1997a). Given these benefits, further screening for suitable fungi which support further development of protocorms to the seedling stage and beyond is vital. Experiments using *in situ* seed baiting techniques as demonstrated by Cruz-Higareda *et al*., (2015), followed by fungal isolation from resulting protocorms and fungal identification would likely lead to the discovery of the most suitable isolates for these species.

Pleurothallid orchids face ever increasing threats due to habitat loss, degradation and removal of plants for the horticultural trade. There is a great need to preserve these and other orchid species, many of which are endangered or worse. It is essential that effective propagation techniques are developed for these species which are most at risk and will benefit from *ex situ* conservation efforts. This study has highlighted important factors in the propagation of Pleurothallid orchids and has demonstrated optimal asymbiotic media which can produce viable plantlets *in vitro*. Further investigations focusing on optimising the abiotic and biotic variables such as seed scarification techniques and times; self-incompatibility of parent plants; seed longevity; asymbiotic media constituents and isolation of fungi from protocorms and seedlings *in situ* could help improve upon these results with all species tested. Assessing the long term data from this study will help gain a better understanding of the effectiveness of treatments and allow for appropriate culture protocols to be established.

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Chapter 7.

A method for using microcosms for the *ex situ* study of tripartite symbioses between ectomycorrhizal plants, fungi and myco-heterotrophic plants

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Abstract

Ex situ study and propagation of myco-heterotrophic orchids poses some difficulties. A method is presented, using microcosms for the initiation of tripartite symbioses with tree seedlings, fungi and orchids. Combinations between tree seedlings of: *Fraxinus excelsior* L.; *Quercus robur* L.; *Tilia cordata* Mill., seed of the myco-heterotrophic orchid *Neottia nidus-avis* (L.) Rich. and soil samples containing fungi from the orchid habitat were attempted. Microcosms consisted of an inorganic culture medium to which was added a soil sample and tree seedling from the *situ* of *N. nidus-avis*, and two seed packets containing seeds of *N. nidus-avis* from two different sources. Associations between tree seedlings, fungi and orchid seed were observed, however, germination was not achieved with seed of *N. nidus-avis* (L.) Rich. after 36 months culture. The method provides a basis which with further refinement could be used for the propagation of myco-heterotrophic and highly fungal dependant mixotrophic orchids. Potentially allowing for the reintroduction of these orchids, complete with symbiotic fungi and ectomycorrhizal plants into suitable habitats. In addition, this method could also see potential application in a range of ecological studies.

Keywords Orchidaceae • Mycorrhizae • Myco-heterotrophy • Microcosm • Symbiotic culture

Introduction

Myco-heterotrophic (MH) orchids rely solely on fungi for their energy source and often have reduced rhizomes and roots. They are mostly achlorophyllous with a few exceptions such as *Limodorum abortivum* (Linne) Sw., which still retains some chlorophyll in its stalk (Blumefeld, 1935; Rasmussen, 2006). Myco-heterotrophic orchids can be considered to represent the most advanced stage of orchid mycotrophy, with many species involved in tripartite relationships with ectomycorrhizal fungi and trees. It is now thought that these orchids are epiparasitic consuming energy and nutrients from both tree and fungus (Leake, 2005; Roy *et al*., 2009a). A broad variety of fungi have been found to associate with MH orchids, including: *Armillaria spp.* (Terashita & Chuman, 1987; Lan *et al*., 1994); Ceratobasidiaceae (Warcup, 1985; Bougoure *et al*., 2009);

Coprinaceae (Yamato *et al*., 2005); Cortinariaceae (Roy *et al*., 2009a); *Erythromyces crocicreas* (Berk. & Broome) (Umata, 1995); Russulaceae (Taylor & Bruns, 1999; Roy *et al*., 2009a); Sebacinaceae (McKendrick *et al*., 2002; Selosse *et al*., 2002; Taylor *et al*., 2003) and Thelephoraceae (Taylor & Bruns, 1997; Roy *et al*., 2009b).

The majority of MH orchids are thought to be highly specific in their choice of fungal partners (Leake, 2004), though some have been shown to associate with a number of fungi, at least during germination (Umata, 1995). Highly fungal dependent mixotrophic (HFDM) orchid species such as *Cephalanthera* (Bidartondo & Read, 2008; Yamato & Iwase, 2008) and *Epipactis spp.* (Bidartondo & Read, 2008; Těšitelová *et al*., 2012) have been found to associate with numerous mycorrhizal fungi. These include fungi from the families: Cortinariaceae (Bidartondo & Read, 2008); Pyronemataceae (Těšitelová *et al*., 2012); Russulaceae (Bidartondo & Read, 2008; Yamato & Iwase, 2008; Těšitelová *et al*., 2012); Sebacinaceae (Bidartondo & Read, 2008; Těšitelová *et al*., 2012); Thelephoraceae (Bidartondo & Read, 2008; Yamato & Iwase, 2008; Těšitelová *et al*., 2012) and Tuberaceae (Bidartondo & Read, 2008; Těšitelová *et al*., 2012). These orchids are thought to be partially myco-heterotrophic (Bidartondo *et al*., 2004; Julou *et al*., 2005; Motomura *et al*., 2010; Yagame *et al*., 2012) and are highly reliant on fungi for the provision of nutrients (Bidartondo *et al*., 2004; Julou *et al*., 2005; Abadie *et al*., 2006), to the extent that when asymbiotic seedlings of *Cephalanthera falcata* (Thunb.) Blume were introduced into a suitable natural habitat, few persisted (Yamato & Iwase 2008).

Myco-heterotrophic orchids have been propagated *ex situ* using symbiotic *in vitro* techniques on agar based media (Umata, 1995, 1998a, 1998b), inorganic soils with a complex carbohydrate source (Yagame *et al*., 2007) and *ex situ* containers containing soil, utilising tripartite associations between *Rhizanthella gardneri* R. S. Rogers, a *Rhizoctonia* fungus (later identified via molecular techniques as Ceratobasidiaceae (Bougoure *et al*., 2009)), and the ectomycorrhizal shrub *Melaleuca uncinata* R. Br (Warcup, 1985). The HFDM orchid, *Cephalanthera falcata* has also been propagated from seed. This was achieved by first using asymbiotic *in vitro* techniques to produce seedlings (Yamato & Iwase, 2008), followed by the initiation of tripartite associations *ex situ*, using containers with inorganic soil, Thelephoraceae fungi and seedlings of

Quercus serrata Murray (Yagame & Yamato, 2012). The *in situ* seed packet method has been used successfully to germinate seeds and produce seedlings of MH (McKendrick *et al.,* 2000a, 2002) and HFDM orchids (Bidartondo & Read, 2008). In the case of McKendrick *et al.,* (2000a) the resultant seedlings of *Corallorhiza trifida* Châtel were removed and trialled in *ex situ* microcosm containers containing soil and tree seedlings from the habitat.

Despite these successes, very few MH and HFDM orchids have been propagated from seed *ex situ*. Methods for propagating these rare and often endangered MH and HFDM orchids (Whigham & Willems, 2003; Cheffings *et al*., 2005; Bougoure *et al*., 2009) have yet to be fully explored. There are no current methods to propagate a wide variety of MH and HFDM orchids to the extent that they can be successfully reintroduced into suitable habitats. Particularly those that interact with ectomycorrhizal fungi and other plants. Such methods could prove useful in maintaining MH and HFDM orchids *ex situ* for conservation reintroduction programmes when species are under threat from habitat loss, degradation and human interference. This work could also help with future scientific studies on multipartite relationships, helping to improve our understanding of the ecology and physiology of MH and HFDM orchids.

The aim of this study was to test whether the MH orchid *N. nidus-avis* could be germinated and grown with a simplified method in *ex situ* microcosms. An additional aim was to test the hypothesis that the presence of ectomycorrhizal plants are necessary to induce germination and development in *N. nidus-avis.* Developing a simplified *ex situ* propagation method for MH and HFDM orchids that allows for successful reintroduction of orchids, complete with their tripartite associations, could assist with future conservation efforts and scientific studies.

Materials and methods

Organismal details

Neottia nidus-avis is found across Eurasia from Britain and Ireland in the west to Iran and western Siberia in the east, there are also isolated populations in western North Africa. In its southern range this species is found at higher altitudes up to 2000 m (Delforge, 2006). It is commonly found on calcareous soils in beech,

hazel, hazel – oak and less commonly in yew and coniferous woodlands, most often in deep shade (Fuchs & Ziegenspeck, 1925; Summerhayes, 1951; Mckendrick *et al*., 2002; Harrap & Harrap, 2009). This species has also been observed in mixed oak – small-leaved lime woodland on neutral to acid soils and mixed coniferous – deciduous woodland (Pers. obs). *Neottia nidus-avis* is a myco-heterotrophic orchid, with almost no photosynthetic pigments other than a small amount of chlorophyll *a* and carotenoids (Montfort & Küsters, 1940; Rasmussen, 2006). It has been found to associate with fungi in Sebacinaceae, especially *Sebacina spp.* which were also found as ectomycorrhiza's on surrounding trees (Mckendrick *et al*., 2002; Selosse et al., 2002).

Soil and tree seedling samples were collected from a mixed oak – small-leaved lime woodland on neutral to acid soils at Goslings Corner Wood, Lincolnshire, U.K. Permissions for soil and tree seedling collection was obtained from Lincolnshire Wildlife Trust and Natural England. Six cylindrical (Ø 40 x 38 mm, length 20 cm) soil samples were taken using an Eijkelkamp soil coring kit (Eijkelkamp, Giesbeek, Gelderland, Netherlands) at random 1 – 3 metre distances from a colony of *N. nidus-avis*. A closer sample could not be obtained due to the absence of fruiting bodies at the time of collection. The soil samples largely consisted of loam interspersed with smaller amounts of organic litter and ectomycorrhizal tree roots, with lower portions of soil cores gradating into clay. Tree seedlings at the site were collected in an attempt to represent the dominant tree species and also provide a source of potential fungal germinators. The forest was largely dominated by *Tilia cordata* Mill.*, Quercus robur* L. and *Fraxinus excelsior* L. Twenty seedlings each, of *F. excelsior*; *Q. robur* and *T. cordata,* varying in height from 10 – 15 cm were collected for the experiment.

Experimental procedure

Mature seeds of *Neottia nidus-avis* were obtained from two sources. Firstly, from the Balkan Rare Plant Nursery (Elena, Veliko Tarnovo, Bulgaria) from plants growing in mixed deciduous woodland on the property. The second was collected from a mixed coniferous-deciduous woodland site to the east of Beckerskichhof, Saale-Holzland-Kreis, Thurinigia, Germany, with permissions from the Untere Naturschutzbehörde im Saale-Holzland-Kreis, see Appendix 1. Both seed provenances were harvested in the summer of 2013. Seeds were stored in

grease proof paper packets which were kept in sealed containers with silica gel at 3±1 ºC until use. Prior to sowing, a triphenyltetrazolium chloride (TTC) seed viability test was performed on seeds from both provenances, as detailed in Chapter 3.

Microcosms were constructed using double Magenta™ GA-7 vessels (Magenta Corp., Chicago, USA). The bottom box was filled with inorganic culture medium similar to those used by Yagame *et al*., (2007) which consisted of an initial drainage layer with 2 cm of Hyuga, a volcanic soil from Miyazaki Prefecture, Japan, on top of which was added 7 cm of culture medium. This consisted of molar, a fired clay product and pumice which was prepared in the ratio of 1:1. Before preparing the microcosms all media were autoclaved and once cooled 5 teaspoons of combined soil sample were mixed into the media as a source of fungal inoculum. A tree seedling was then planted in each magenta and cut to size where appropriate. Any resulting wounds were dusted in charcoal powder in order to help prevent pathogenic infection of the tree seedlings. Twenty replicate containers were prepared for each tree species with an additional twenty containers set up as non-ectomycorrhizal plant controls, giving a total of eighty containers.

Seed packets were prepared using 35 mm plastic photographic slide mounts with a 10 x 5 cm piece of 48 micron nylon mesh (Plastok Associates Ltd., Birkenhead, UK) in a similar manner to Rasmussen & Whigham (1993), Brundrett *et al.,* (2003) and Batty *et al*., (2006b). 100 – 200 seeds were placed on the mesh, the mesh was then folded over and sandwiched inside the slide. Two seed packets**,** one from each provenance**,** were placed in each container at opposite sides of the magenta vessel. They were placed against the sides of the vessel approximately 2 cm under the compost surface, allowing for viewing of the mesh from the sides of the vessel**.** Deionised water was added to moisten compost until water could be seen pooling to a depth of no more than 1 cm in the drainage layer. The second magenta vessel was then added over the top of tree seedlings to seal the microcosm and a layer of aluminium foil was wrapped around the bottom magenta to exclude light from the culture media (Figure 1a).

Microcosms were set-up on 08/11/2013 and were placed in a lit plant growth cabinet Sanyo MLR-350 (Sanyo Electric Co. Ltd., Osaka, Japan) under

Goodlight LED T8 20 W lights (LED Eco Lights Ltd., Camberley, U.K.) at 40 µmol m^{-2} s^{-}1. In order to recreate the natural climate cycles which the study species receive, a simplified temperate temperature and photoperiod regime was implemented. The microcosms first received a 3 month cold winter period at 3±1 ºC with a photoperiod of 8:16 h light/dark, followed by a 9 month warm period 20±1 ºC with a photoperiod of 16:8 h light/dark. Temperatures were gradually decreased/increased over the course of the first month of each temperature treatment until the desired temperature was reached. After one year the cycle was repeated. Moisture content of the vessels was checked visually on a monthly basis and supplemented with deionised water where necessary. Cultures were fertilised during the warm growth period three times at intervals of three months with 2 ml of quarter strength Hoagland's solution (Hoagland & Arnon, 1950) during the warm growing period. After 18 and 36 months all seed packets were checked for germination using a stereomicroscope (Leica M275, Leica Microsystems, Milton Keynes, UK).

Results

Embryo counts revealed the presence of embryos in seeds of both provenances of *N. nidus-avis*: 84.33% for Bulgarian and 87% with German seed. Viability staining using TTC resulted in staining in both provenances, with 48.67% in Bulgarian and 53.22% in German seed.

After 18 and 36 months culture, no germination was observed in seed packets in any treatments. Despite this, associations between tree seedling roots, fungi and orchid seed were observed from 18 months culture with all tree species. Fungal hyphae were observed to be interacting and emanating from the roots of tree seedlings which were outside the seed packet (Figure 1b), and in some cases where they had penetrated the nylon mesh of the seed packet. These hyphae were also observed to be interacting with *N. nidus-avis* seed, either as masses of hyphae surrounding seeds (Figure 1c) or as individual hyphae penetrating the seed testa (Figure 1d). Differing fungal communities were observed between tree species treatments. Treatments with *F. excelsior* only appeared to have a fungus with white hyphae which interacted with both *F. excelsior* roots and formed masses of hyphae around *N. nidus-avis* seed, as seen

with *Q. robur* (Figure 1b) and *T. cordata* (Figure 1c). Treatments with *Q. robur* and *T. cordata* appeared to have a higher diversity of fungi associating with both tree roots and *N. nidus-avis* seed. In addition to the white fungal hyphae that were found in *F. excelsior* treatments, both brown and dark (black) fungal hyphae were observed associating with both roots of *Q. robur* and *T. cordata* and penetrating the testa of *N. nidus-avis* seed. In contrast to the observations with the white fungal hyphae, the brown and dark (black) hyphae were found at much lower densities, in both seed packets and when associating with *N. nidus-avis* seed. Closer microscopic observations of the dark (black) hyphae, revealed similarities to dark septate endophytes such as *Leptodontidium, Phialocephala* and *Phialophora spp*. It should be noted, as no germination occurred, the confirmation of mycorrhizal associations via staining was not conducted and the identities of associating fungi were not ascertained.

Fungal hyphae were observed inside seed packets of non-ectomycorrhizal plant controls from 18 months culture, but were not actively interacting with *N. nidus-avis* seed as observed in treatments with tree seedlings. Some tree seedling death was observed, especially with seedlings of *Q. robur* (7/20) compared to (2/20) in *F. excelsior* and none in *T. cordata* treatments. Death of *Q. robur* seedlings may have occurred due to the trimming of a number of larger seedlings so they would fit in the containers. Despite treatment of wounds with charcoal powder, seedlings still succumbed to fungal infection which may have been compounded by the lack of air exchange due to the nature of the sealed containers. In addition to the intended organisms, a diversity of other organisms were found in microcosm containers which originated from the original soil sample. These included mosses, grasses, sedges, ferns and herbs which required removal on a regular basis. A number of different Collembola were also observed.

Figure 1. a) Double Magenta® GA-7 vessels, each containing two seed packets of *Neottia nidus-avis* seed, with and without *Quercus robur* seedlings, showing aluminium foil shielding around the bottom vessel containing compost, at 18 months culture. **b)** 35 mm plastic photographic slide mount with 48 micron nylon mesh seed packet containing *Neottia nidus-avis* seed, submerged under the compost and viewed from side of culture box, after 18 months culture with *Quercus robur*. Note: root of *Quercus robur* and white fungal hyphae interacting with root and seed packet mesh. **c)** *Neottia nidus-avis* seed of Bulgarian origin, inside a seed packet after 36 months culture with *Tilia cordata*. Black arrows indicate brown fungal hyphae and red arrows indicate a mass of clear fungal hyphae. Both are interacting with *N. nidus-avis* seed and roots of *T. cordata* outside the packet (scale bar = 200 µm). **d)** *Neottia nidus-avis* seed of German origin inside a seed packet from a different replicate after 36 months culture with *Tilia cordata*. Red arrows indicate dark fungal hyphae that are interacting with *N. nidus-avis* seed and roots of *T. cordata* outside the packet (scale bar = 200 µm).

Discussion

Germination in *N. nidus-avis* did not occur after 36 months culture, despite TTC tests showing around half of the tested seeds were viable. As a result the hypothesis that the presence of ectomycorrhizal plants are necessary to induce germination and development in *N. nidus-avis* could not be proved. Associations were, however, observed between tree seedling roots, fungi and orchid seed after 12 months culture. There appeared to be differences in the fungal communities of tree species treatments, with a higher number of fungal morphospecies forming associations between tree seedling roots and orchid seed in *Q. robur* and *T. cordata* treatments. This indicated that seedlings of these tree species had either higher diversities of associating and potentially mycorrhizal fungi prior to the experiment, or that they were able to form these associations after the experiment had commenced. Interestingly, these observations do somewhat reflect the relative fungal diversities observed in these tree species. A study examining the diversity of ectomycorrhizal fungi in Britain (Newton & Haigh, 1998) found only two fungal species associating with *Fraxinus spp.,* none of which were ectomycorrhizal. In comparison, *Tillia spp.* were found to associate with 33 fungal species, two of which were ectomycorrhizal. *Quercus spp*. displayed some of the highest fungal diversity, associating with 233 fungal species, 30 of which were ectomycorrhizal. It should be noted that *Fraxinus spp.* have largely been found to associate with arbuscular mycorrhiza (Harley & Harley, 1987).

Three different morphospecies of fungi were observed interacting with tree seedling roots and seed of *N. nidus-avis.* Firstly, a fungus with white hyphae which was observed across all three tree species treatments and formed masses of hyphae around *N. nidus-avis* seed. The second, formed brown fungal hyphae and the third, had dark (black) hyphae, both of which only occurred in treatments with *Q. robur* and *T. cordata*. The brown and dark (black) hyphae were regularly observed penetrating into the testa of *N. nidus-avis* seed. There was no observable difference in embryo swelling or degradation between 'uninfected' seeds and those with hyphal penetration. Therefore, it could not be determined whether these associations were as a result of potential symbiotic, saprotrophic or random fungal growth.

Observations of the dark (black) hyphae revealed similarities to dark septate endophytes (DSE) such as *Leptodontidium, Phialocephala* and *Phialophora spp*. It should be noted, however, that molecular analysis of DSE's has shown them to be of diverse origins (Jumpponen & Trappe, 1998a). Dark septate endophytes such as *Leptodontidium, Phialocephala* and *Phialophora spp*. have been isolated from chlorophyllous orchids (Currah *et al*., 1987, 1988, 1997; Bidartondo *et al*., 2004; Hou & Guo, 2009), and found to germinate chlorophyllous terrestrial orchids (Chapter 2; Zimmerman and Peterson, 2007). They have also been found associating with herbs (Fernando & Currah, 1996) and trees (Wang & Wilcox, 1985; Wilcox & Wang 1987a, 1987b; Jumpponen & Trappe, 1998a, 1998b). Although DSE's have not been found associating with *N. nidus-avis, Leptodontidium orchidicola* Sigler & Currah has been found associating with germinating seed of the HFDM orchid *Cephalanthera damasonium* (Mill.) Druce (Bidartondo & Read, 2008). *Phialophora* and *Leptodontidium* have also been identified as mycorrhizal fungi with the HFDM orchid genera *Cephalanthera* and *Epipactis* (Bidartondo *et al*., 2004; Bidartondo & Read, 2008). This suggests that although suitable fungi may not have been present to initiate germination in *N. nidus-avis*, this method did allow for the culture of potentially useful orchid-mycorrhizal fungi.

McKendrick *et al.,* (2002), using an *in situ* seed packet technique, found that germination often took place in the presence of a rhizomorph-forming fungus that varied from black-brown to bright orange-brown in colour. These fungi were observed infecting seeds and were often found on the testa of uninfected seeds in which germination had reached the imbibition stage. Subsequent molecular analysis revealed the fungi found in *N. nidus-avis* seedlings and plants to mostly be *Sebacina spp*. (McKendrick *et al.,* 2002). Although the descriptions of these fungi do not match those of the fungi observed in this experiment, the brown and dark (black) hyphae that were observed here, similarly interacted with *N. nidus-avis* seed but did not appear to be at the same hyphal densities as those observed by McKendrick *et al.,* (2002).

Soil samples were collected in proximity to an adult colony of *N. nidus-avis* and contained a number of ectomycorrhizal tree root sections. It was hoped that these samples would contain suitable fungi that could form mycorrhizal associations between tree seedlings and orchid. Resulting in germination and

further development of *N. nidus-avis* germinants. The lack of germination in this study may likely be due to the lack of suitable fungi in microcosm treatments. A number of studies successfully achieved germination and development of MH (Warcup, 1985; Umata, 1995, 1998a, 1998b; Yagame *et al*., 2007) and HFDM (Yamato & Iwase, 2008; Yagame & Yamato, 2012) orchids *ex situ*. In most cases fungi were isolated from the study orchid species with several fungal strains used in germination trials. Prior to beginning this experiment, numerous failed attempts were made to find inflorescence of *N. nidus-avis* at the Goslings Corner Wood site. It is likely that the often irregular nature of inflorescence production in *N. nidus-avis* or presence of excessive grazing may have been to blame. As a result fungi could not be isolated from adult plants and seeds were sourced from other sites across Europe. Selosse *et al*., (2002) showed that there were regional variations in *N. nidus-avis* fungal symbionts. Whether this can also affect the fungal specificity of different populations during germination and development is unknown, and may have played a role in the negative results of this study.

Considering that these microcosms may contain a broad diversity of soil microorganisms and microfauna, it is likely that this environment would better simulate the environment that is experienced by orchid seed in a natural site. With further refinement and development, this method may prove to be a better representation of the soil communities and their interactions than a stricter experimental approach focusing on aseptic techniques for the introduction of species. These complex soil communities may not necessarily assist with optimising the propagation of these orchids, as the complexity of potential abiotic and biotic factors affecting germination may be increased. A number of different Collembola were observed across microcosm treatments. Given that fungi can contribute a major proportion of their diets (Hopkin, 1997), they may have had a negative effect on hyphal densities within the media. A number of other variables such as the soil media, microcosm volume, moisture levels, temperature cycle and the lack of ventilation may have had an adverse effect on forming successful tripartite symbioses. Nevertheless, further optimisation of these factors could improve this method, and may result in the discovery of factors affecting germination and development of these orchids which have yet to be investigated.

Despite, the lack of germination observed in *N. nidus-avis*, the possibilities for the long term maintenance of microcosms with tree seedlings, fungi and soil

communities have been demonstrated. Fungal hyphae were observed to be interacting with tree roots and orchid seed, potentially forming mycorrhizal connections between orchid seed and tree roots. The study highlights a simple method which with further refinement and development may provide a basis for the propagation of MH and HFDM orchids, as well as other MH plants. Further experiments could focus on the isolation of suitable orchidaceous and ectomycorrhizal fungi from different life stages of MH and HFDM orchids, and surrounding ectomycorrhizal plants. The efficacy of the isolated fungi could be tested by inoculating them onto aseptically grown tree seedlings planted in sterile media. Suitable orchid seed packets or seedlings could then be added to the culture media, further cultured and germination and growth assessed. This could allow for the reintroduction of these plants, complete with symbiotic fungi and ectomycorrhizal plants into suitable habitats. This method could also see potential application in a range of ecological studies, exploring species interactions and the effects of abiotic and biotic factors. Developing effective methods to investigate and conserve MH and HFDM orchids is an interesting topic and deserves greater research.

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Appendix

Appendix 1. Collection permit for *Corallorhiza trifida* and *Neottis nidus-avis* from the Untere Naturschutzbehörde im Saale-Holzland-Kreis, Thuringia, Germany.

SAALE-HOLZLAND-KREIS
LANDRATSAMT

Landratsamt · Postfach 1310 · 07602 Eisenberg

Faculty of Life Sciences
The University of Manchester
Oxford Road
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Umweltamt
Untere Naturschutzbehörde

Auskunft erteilt: Herr Günther
Telefon: 036691 70-304
Fax: 036691 70-716
E-Mail: umwelt@lrashk.thueringen.de

Bedingungen zur Nutzung unserer elektronischen Postzugänge siehe: www.saaleholzlandkreis.de

Bei persönlicher Rücksprache:
Eisenberg, Schloßgasse 17, Zi.: 211

Ihr Zeichen	Ihr Schreiben vom	Unsere Zeichen/AZ	Datum
		67.2.1/gü/364.6.622-05/13	2013-06-10

Vollzug des Gesetzes über Naturschutz und Landschaftspflege (Bundesnaturschutzgesetz – BNatSchG) vom 29. Juli 2009 (BGBl. Teil I Nr. 51, S. 2542), zuletzt geändert durch Artikel 5 des Gesetzes vom 6. Februar 2012 (BGBl. I S. 148)

Antrag auf Erteilung einer artenschutzrechtlichen Ausnahmegenehmigung gemäß § 45 Abs. 7 BNatSchG und einer naturschutzrechtlichen Genehmigung für die Durchführung von wissenschaftlichen Untersuchungen im Geltungsbereich

hier: Entnahme von Korallenwurz (Corallorhiza trifida) und Vogel-Nestwurz (Neottia nidus-avis)

Sehr geehrte Damen und Herren,

das Landratsamt des Saale-Holzland-Kreises erlässt folgenden

Bescheid
I.

1. Ihnen wird antragsgemäß eine artenschutzrechtliche Ausnahmegenehmigung gemäß § 45 BNatSchG von den Verboten des § 44 Abs.1 Ziffer 2 BNatSchG für die einmalige Entnahme von max. 5 Pflanzen, je Art Korallenwurz (Corallorhiza trifida) und Vogel-Nestwurz (Neottia nidus-avis), im Waldgebiet östlich der Ortschaft Beckerskirchhof und nördlich der „Hohe Straße" erteilt.

II. Hinweis

Die Erlaubnis ersetzt nicht die gegebenenfalls nach anderen Rechtsvorschriften notwendigen Erlaubnisse und Genehmigungen.

III. Nebenbestimmung

1. Auflagen:

1. Das Vorhaben ist unter größtmöglicher Schonung der Pflanzen- und Tierwelt durchzuführen. Die Entnahme ist auf das für die Untersuchung notwendige Maß zu beschränken und darf nicht 5 Pflanzen je Art Korallenwurz (Corallorhiza trifida) und Vogel-Nestwurz (Neottia nidus-avis) übersteigen.

2. Die Arbeiten im Rahmen der Untersuchung dürfen nur von fachkundigen Personen durchgeführt werden.

3. Zum Jahresende sind die fundortbezogenen Daten entsprechend den Vorgaben der TLUG (Artenerfassungsbögen) zusammen mit einem Ergebnisbericht und einer Fotodokumentation der Unteren Naturschutzbehörde des Saale-Holzland-Kreises unaufgefordert zur Verfügung zu stellen. Die Daten werden dann unsererseits an die TLUG zur Eingabe in das landesweite Landschaftsinformationssystem (LINFOS) weitergeleitet.

4. Aus dem Ergebnisbericht sollen folgende Angaben hervorgehen: durchgeführte Arbeiten, Untersuchungsmethodik, Untersuchungsort sowie Zusammenfassung der Untersuchungsergebnisse.

5. Andere Tiere und Pflanzen dürfen bei der Durchführung des Vorhabens weder beunruhigt oder verletzt werden, noch sonstige Beeinträchtigungen erleiden.

6. Der Bescheid oder eine Kopie davon ist stets im Gelände mitzuführen.

2. Fristen:

Dieser Bescheid gilt bis **31.12.2013**

IV. Begründung:

Mit Schreiben vom 12.03.2013 beantragten Sie bei der Unteren Naturschutzbehörde des Saale-Holzland-Kreises eine Ausnahmegenehmigung von den Verboten des § 44 BNatSchG, für die Entnahme und die Inbesitznahme von 5 Pflanzen der Arten Korallenwurz (Corallorhiza trifida) und Vogel-Nestwurz (Neottia nidus-avis)
Der Antragssteller beabsichtigt eine Methode zu entwickeln zur weiteren Verbreitung dieser o.g. Arten. Erwogene Alternativlösungen dieser Untersuchungsmethode waren nicht vorhanden.

Die (Corallorhiza trifida) und Vogel-Nestwurz (Neottia nidus-avis) sind gemäß Bundesnaturschutzgesetz besonders geschütze Pflanzenarten.

Gemäß § 44 Abs. 2 (2) BNatSchG ist es ferner verboten, Tiere und Pflanzen der streng geschützten Arten in Besitz oder Gewahrsam zu nehmen oder zu haben oder sie zu be- oder verarbeiten (Besitzverbote).

Nach § 45 Abs. 7 BNatSchG und nach § 4 Abs. 3 BArtSchV können die nach Landesrecht zuständigen Behörden von den Verboten des § 44 BNatSchG und § 4 Abs. 3 BArtSchV im Einzelfall

weitere Ausnahmen zulassen, soweit dies für Zwecke der Forschung, Lehre, Bildung oder Wiederansiedlung oder diesen Zwecken dienende Maßnahmen der Aufzucht oder künstlichen Vermehrung erforderlich ist und soweit sich der Erhaltungszustand einer Art nicht verschlechtert, soweit Artikel 16 Absatz 1 der Richtlinie 92/43/EWG und Artikel 9 Absatz 2 beachtet sind und Vorschriften einer Rechtsverordnung nach § 54 BNatSchG, sonstige Belange des Artenschutzes oder Verpflichtungen aus internationalen Artenschutzübereinkommen nicht entgegenstehen.

Die artenschutzrechtliche Ausnahmegenehmigung konnte im genehmigten Umfang erteilt werden, da die Untersuchungen zu Forschungszwecken dienen. An den Untersuchungen besteht erhebliches öffentliches Interesse, da sie wissenschaftliche Grundlagendaten über Verbreitung, Vorkommen und Ökologie erbringen und damit eine wertvolle Arbeitsgrundlage für die Naturschutzbehörden darstellen.

Die artenschutzrechtliche Ausnahmegenehmigung konnte gemäß § 36 Abs. 2 ThürVwVfG mit Nebenbestimmungen versehen werden. Diese waren zum Schutz von Pflanzen und Tieren in den betroffenen Bereichen erforderlich.

Der Auflagenvorbehalt ermöglicht die nachträgliche Aufnahme und Änderung erforderlicher Auflagen.

Bei Missachtung der Nebenbestimmungen kann von dem Recht des sofortigen Widerrufs Gebrauch gemacht werden.

Die Zuständigkeit für die Erteilung einer artenschutzrechtlichen Ausnahme ergibt sich aus § 3 Abs. 1 BNatSchG i.V.m. § 29 Abs. 1 und § 36 Abs. 4 ThürNatG. Die untere Naturschutzbehörde des Saale-Holzland-Kreises ist örtlich und sachlich zuständig. Die örtliche Zuständigkeit folgt aus § 3 des Thüringer Verwaltungsverfahrensgesetzes (ThürVwVfG) in der Fassung der Bekanntmachung vom 18. August 2009 (GVBl. S. 699), zuletzt geändert durch Artikel 10 des Gesetzes vom 9. September 2010 (GVBl. S. 291).

Die Kostenentscheidung beruht auf Ziffer I. der Anordnung des TMLNU vom 20.04.2009: „Anordnung zum Absehen der Erhebung von Verwaltungskosten für bestimmte Arten von Leistungen". Demnach ist von der Erhebung von Verwaltungsgebühren für Ausnahmegenehmigungen nach § 43 Absatz 8 Satz 1 Nr. 2, für Befreiungen gemäß § 67 BNatSchG und Anordnungen gemäß 3 BNatSchG und § 32 Abs. 1 ThürNatG unter der Voraussetzung abzusehen, dass die Datenerfassung nicht gewerblichen Zwecken dient und die gewonnenen Daten der Naturschutzverwaltung zur Verfügung gestellt werden. Im vorliegenden Fall kommt die Untere Naturschutzbehörde zu dem Ergebnis, dass die geforderten Voraussetzungen erfüllt sind. Demzufolge wird von einer Gebührenerhebung abgesehen. Besondere Auslagen sind nicht angefallen.

V. Rechtsbehelfsbelehrung

Gegen diesen Bescheid kann innerhalb eines Monats nach seiner Bekanntgabe Widerspruch erhoben werden. Der Widerspruch ist schriftlich oder mündlich zur Niederschrift beim Landratsamt Saale-Holzland-Kreis, Im Schloß, 07607 Eisenberg einzulegen.

i.V.
Schirmer
Amtsleiter

Chapter 8.

General Discussion

The work presented in this thesis focuses on orchid-mycorrhizal relationships and its application to the propagation of epiphytic and terrestrial orchids. These studies have added to our understanding of orchid-mycorrhizal relationships, highlighting important factors that affect orchid germination and development *in vitro*. Effective methods for *in vitro* germination and development have been demonstrated for a number of orchid species. These will assist future propagation of orchids for conservation, horticulture and scientific studies.

The orchid-mycorrhiza relationship, plays a key role in orchid reproduction; with fungi, first initiating orchid seed germination, followed by development of protocorms and seedlings, to the establishment of adult plants (Rasmussen, 2006). Fungal specificity is a significant factor in the distribution of orchids and the colonisation of habitats. In Chapter 2, *in vitro* fungal compatibility during germination and development was investigated in the Southwest Australian terrestrial orchid, *Microtis media* R. Br. This was achieved by sowing seeds on symbiotic H1 oat media (Clements *et al*., 1986, modified by Rasmussen *et al*., 1990a) with five fungi from orchid and non-orchid sources. These fungi had, either; similar or differing geographic distributions to *M. media* and comprised: *Ganoderma australe* (Fries) Patouillard complex, *Phialocephala fortinii* C. J. K.Wang & H. E.Wilcox, *Rhizoctonia repens* N. Bernard, *Rhizoctonia solani* Kühn and *Sebacina vermifera* Oberwinkler.

Microtis media demonstrated very low fungal specificity in germination, with all five fungal isolates initiating germination. Later development, however, was more specific, with only one fungus, a non-orchid strain of *R. solani* isolated from *Brassica oleracea* L. in Belgium, proving most compatible by inducing protocorm development through to the formation of adult plants. The study found new symbiotic interactions between the Southwest Australian terrestrial orchid *M. media* and fungi with differing geographic distributions from the orchid: *R. repens* and non-orchid sources: *G. australe*; *P. fortinii* and *R. solani*. This demonstrates,

in this case, that orchid/fungal distribution and fungal origin are no barrier to inducing successful orchid-fungal symbioses.

Terrestrial orchids are commonly propagated from seed using agar based nutrient media which can be symbiotic, where a suitable fungus is cultured in association with orchid seed, or asymbiotic, where seeds are sown without fungus on a sterile nutrient media. Fungal compatibility is an important factor in the symbiotic germination and development of orchids. In order to determine whether there were differences in fungal compatibility among species of an orchid subtribe, in Chapter 3 fungal compatibility was tested in four Eurasian terrestrial orchids from the subtribe Orchidinae. These were: *Anacamptis morio* (L.) R.M.Bateman, Pridgeon & M.W.Chase, *Dactylorhiza incarnata* (L.) Soó, *Serapias bergonii* E.G.Camus, *Serapias parviflora* Parl. Seeds of these orchids were sown on H1 symbiotic media with five fungi, two species of the well-known orchid symbiont genus *Rhizoctonia*: *Rhizoctonia repens* N.Bernard; *Rhizoctonia solani* Kühn and three strains of mycorrhizal fungi isolated from terrestrial orchids: A36; B1 (*Ceratobasidium sp.* Anastamosis Group C), isolated from *Dactylorhiza fuchsii* (Druce) Soó; Q414, isolated from *Dactylorhiza iberica* (M.Bieb. ex Willd.) Soó; and an asymbiotic control using Malmgren (1996) modified media (Malmgren & Nyström, 2014).

Varying degrees of fungal specificity were observed in the tested orchid species during germination and development. *Dactylorhiza incarnata* and *A. morio* displayed the lowest fungal specificity, with *D. incarnata* germinating on all treatments, and B1 proving most successful in germination and development. *Anacamptis morio* germinated on all treatments with the exception of *R. solani*, with the most advanced development with the fungi: A36 and B1. *Serapias bergonii* and *S. parviflora* were more specific. *Serapias bergonii* only germinated with the fungi *R. repens* and Q414, and *S. parviflora* only with the fungus Q414. Both species germinated most successfully on the asymbiotic control, though the fungus Q414 resulted in the most advanced development. This study has highlighted the differences in fungal specificity between different orchid species within the orchid subtribe Orchidinae. These observations may be reflected in the recruitment behaviour and habitat choice of the different orchid species. Species with low fungal specificity such as *D. incarnata* could be more generalist in their habitat choice, potentially associating with a broad web of fungi. Whereas species

displaying high fungal specificity such as *Serapias spp.* may colonise niche habitats associating with narrower webs of fungi. Effective propagation methods and fungal partners for producing seedlings of a number of Eurasian tuberous terrestrial orchids have been demonstrated. These results have implications for future propagation of terrestrial orchids for conservation and horticulture.

Research into symbiotic and asymbiotic *in vitro* methods on the germination of orchids has received notable attention by researchers. Little comparative work, however, has been conducted to ascertain their effectiveness at producing viable plants and determine their survival *ex vitro*. In Chapter 4, a study was conducted to determine the effectiveness of asymbiotic and symbiotic *in vitro* propagation methods, with two European terrestrial orchid species with potential commercial importance: *Anacamptis laxiflora* (Lam.) R. M. Bateman, Pridgeon & M. W. Chase and *Ophrys apifera* Huds. Both orchid species were sown on asymbiotic Malmgren modified media and symbiotic H1 media. On symbiotic media, *Anacamptis laxiflora* was sown with the B1 fungal isolate (*Ceratobasidium sp*. Anastamosis Group C), isolated from *Dactylorhiza fuchsii* (Druce) Soó, whilst *O. apifera* was sown with an unidentified FOA8 fungus isolated from *O. apifera*.

Symbiotic methods resulted in higher germination and development with both orchid species. A significantly higher number of *A. laxiflora* germinants were obtained with the symbiotic method, with around three times more germinants than the asymbiotic method. Germination in *O. apifera* was far lower in comparison to those achieved with *A. laxiflora*. Symbiotic culture gave the highest overall germination in *O. apifera*, with almost double the relative number of germinants compared to the asymbiotic method. Symbiotic germination in *O. apifera* was far more variable across plates, with some plates exhibiting large numbers of germinants and others none at all. Asymbiotic germination in comparison was far more uniform across plates.

Development in both orchids was more advanced in symbiotic cultures with *A. laxifolia* producing leaves and roots and *O. apifera* forming protocorms with rhizoids after 2 months culture. Development of *O. apifera* largely arrested after 3 months on both treatments. At 18 months of culture, the symbiotic method had produced more than double the number of symbiotic *A. laxiflora* plantlets,

compared to asymbiotic plantlets. After 30 months culture, 12 months of which were *ex vitro*, no asymbiotic ex-plants had survived. However, more than 70% of symbiotic ex-plants survived with some asexual propagation taking place amongst larger plants. The results of this study demonstrate the utility and advantage of symbiotic techniques for the propagation of orchid species. Assessing and determining optimal propagation methods for various orchid genera and species can help improve the success and efficiency of orchid propagation for commercial and conservation purposes. A future investigation to unequivocally determine fungal symbiosis in this and other studies could be performed by sectioning and re-isolating the fungus from plant root tissues. Additional symbiotic sowing tests could be conducted to allow for sectioning and isolation of the tested fungi from orchid protocorms. The re-isolated fungi's efficacy could then be tested in further sowing experiments, potentially leading to the identification of optimal fungal isolates and improving our understanding of orchid-mycorrhizal relationships.

A number of abiotic and biotic factors may affect orchid germination and development. Observations in Chapters 2; 3 and 4, have shown varied and sometimes unusual germination and developmental distributions of terrestrial orchids across symbiotic treatments on agar based media *in vitro*. In order to further investigate observations made in Chapter 4, a study was conducted to determine whether there was an effect of substrate rugosity and complexity on the germination and development in symbiotic cultures of terrestrial orchids. In Chapter 5, I investigated this effect on two Eurasian terrestrial orchids: *Anacamptis morio* and *Dactylorhiza purpurella* (T.Stephenson & T.A.Stephenson) Soó. In order to investigate the effect of substrate rugosity on germination and development, seeds of these orchids were sown on agar based media with either flat or cut treatments. To determine whether substrate complexity had an effect; inorganic and soil based media were also tested. These media treatments were split into asymbitoic and symbiotic treatments using either asymbiotic Malmgren modified media or symbiotic H1 media with the B1 fungal isolate (*Ceratobasidium sp*. Anastamosis Group C).

The study showed that increased substrate rugosity on agar-based media had a positive effect on germination and development in symbiotic cultures of *A. morio* and both asymbiotic and symbiotic cultures of *Dactylorhiza purpurella*. The

effect of the method on agar based cultures was also highly significant in *D. purpurella* with higher germination in symbiotic treatments. The presence of a fungal symbiont increased the magnitude of germination overall, but when there were cuts in the media this effect was magnified. This suggests that the magnitude of the rugosity effect in both orchid species was dependent on whether a symbiont was present. *Anacamptis morio* showed a significant response to substrate complexity upon germination in symbiotic cultures across all treatments. *Dactylorhiza purpurella* in contrast, showed a significant effect of substrate complexity across treatments in both asymbiotic and symbiotic methods. The results of this study show that substrate rugosity and complexity are important factors in the germination and development of these terrestrial orchids *in vitro*. Whether these observations are an effect of substrate complexity on the fungus or a direct effect of substrate or both depends on the species of orchid. Further research is needed to help better determine its implications in orchid-fungal interactions, their symbiosis and orchid propagation.

Pleurothallidinae is one of the largest subtribes in Orchidaceae, representing more than 5000 species (Karremans, 2016). These Neotropical orchids are mostly comprised of epiphytes with a number of lithophytic, terrestrial and even rheophytic species (Pridgeon, 1982; Pridgeon *et al*., 2001; Higgins & Williams, 2009). Many of these orchids are considered rare, threatened or endangered (Llamozas *et al*., 2003; Calderón-Sáenz, 2006; Millner, 2013). This is largely due to species often exhibiting narrowly restricted populations that occupy niche habitats with low numbers of individuals (Tremblay & Ackerman, 2001; Jost, 2004; Crain & Tremblay, 2012, Pérez-Escobar *et al*., 2013; Pérez-Escobar *et al*., 2017). In such cases even minor changes to their habitat can have negative impacts on their populations (Cribb et al., 2003; Swarts & Dixon, 2009b). Considering their plight, the existing literature on the propagation of these orchids is very sparse. Resultantly, little is known of factors affecting their germination and development, or their developmental biology as a whole in this respect.

In Chapter 6, a study was conducted to help determine optimal propagation methods for six species from four genera of the orchid subtribe, Pleurothallidinae: *Masdevallia melanoxantha* Linden & Rchb.f.; *Masdevallia menatoi* Luer & R.Vásquez*; Masdevallia patchicutzae* Luer & Hirtz*; Pleurothallopsis microptera* (Schltr.) Pridgeon & M.W.Chase*; Restrepia contorta*

(Ruiz & Pav.) Luer; *Stelis sp*. Peru. Seeds were sown using both asymbiotic and symbiotic methods. Asymbiotic sowings were made with all six species on half strength and, in the case of *M. melanoxantha*; *M. menatoi*; *P. microptera,* full strength asymbiotic Knudson C (Knudson, 1946), Sigma P6668 – Phytamax™ orchid maintenance medium and Vacin and Went (VW) (Vacin & Went, 1949) media. For *M. melanoxantha,* additional treatments containing 100 ml l^{-1} of coconut water were included with half and full strength asymbiotic media. Symbiotic sowings were made on H1 media with three fungi isolated from roots of the Pleurothallid orchids: *Dracula felix* (Luer) Luer; *Lepanthopsis astrophora* (Rchb.f. ex Kraenzl.) Garay; *M. menatoi* which were growing *ex situ* in the National Plant Collection® of Pleurothallid orchids.

There was a significant effect of treatment on all orchid species. Germination amongst the tested orchid species was highest with two fungal isolates: Df, isolated from *D. felix* and Mm, isolated from *M. menatoi*, with the fungal isolate Mm proving most successful across species. Germination on asymbiotic media was low, compared to these symbiotic treatments, with certain species showing preferences for certain asymbiotic media formulations during germination. Asymbiotic media strength and coconut water did not have a significant effect on germination. Asymbiotic germination on all variations of Sigma P6668 media resulted in advanced development, forming plantlets with leaves and in some cases roots. Development on the symbiotic treatments and on the asymbiotic Knudson C treatment, however, did not progress beyond early stage protocorms. This study demonstrates an initial advantage for symbiotic germination, but advanced development occurred only on one asymbiotic media. Isolation of fungi using *in situ* seed baiting methods as used by Cruz-Higareda *et al*., (2015) and isolation from plant roots *in situ* may help with the discovery of more suitable fungal symbionts. These results show that there is some species specific preferences for certain media formulations during germination, however, subsequent development was largely supported on one media formulation. Such findings help to improve our knowledge of factors affecting their germination and development of these orchids. Potentially assisting the production of Pleurothallid orchids for conservation and horticulture.

Ex situ studies of myco-heterotrophic (MH) and highly fungal dependent mixotrophic (HFDM) orchids poses some difficulties. This is largely due to their

intrinsic associations with fungi and ectomycorrhizal plants (Rasmussen, 2006). Myco-heterotrophic and highly fungal dependent mixotrophic orchids have been propagated symbiotically *ex situ* using a number of methods (Warcup, 1985; Umata, 1995, 1998a, 1998b; McKendrick *et al.,* 2000a, 2002; Yagame *et al*., 2007; Yamato & Iwase, 2008; Yagame & Yamato, 2012). Although a broad range of methods have been used to propagate MH orchids. None of these methods have been used to germinate a wide range of MH and HFDM orchids, to the extent that they can be successfully reintroduced.

In Chapter 7, I demonstrate a simple method using *ex situ* microcosm containers for the potential initiation and investigation of tripartite symbioses between tree seedlings, fungi and orchids. Associations were attempted between tree seedlings of: *Fraxinus excelsior* L.; *Quercus robur* L.; *Tilia cordata* Mill., seed of the MH orchid *Neottia nidus-avis* (L.) Rich. and soil samples containing fungi from the orchid habitat. Microcosms were constructed using double Magenta® GA-7 Plant Culture Boxes. The bottom box was filled with inorganic culture medium, composed of sterilised volcanic soils and fired clay media. To this was added a soil sample and tree seedling obtained from the situ of *N. nidus-avis* plants, and two seed packets containing seeds of *N. nidus-avis,* each from a different source. Associations between tree seedling roots, fungi and orchid seed were observed after 12 months culture. After 36 months culture, no germination was observed in seed of *N. nidus-avis*, despite the observation of close interactions between fungi and seeds.

The study highlights a simple method which, with further refinement and development, may provide a basis for the propagation of MH and HFDM orchids. Further experiments could be conducted using this method, focusing on isolating suitable orchidaceous and ectomycorrhizal fungi from different life stages of MH and HFDM orchids surrounding ectomycorrhizal plants. The efficacy of these fungi could be tested by inoculating them onto aseptically grown tree seedlings planted in sterile media. Suitable orchid seed packets or seedlings could then be added, further cultured and germination and growth assessed. If successful, this could allow for the reintroduction of these orchids, complete with symbiotic fungi and ectomycorrhizal plants into suitable habitats. This method could also see potential application in a range of ecological studies, exploring species interactions and the effects of abiotic and biotic factors.

I have shown that fungal specificity and compatibility *in vitro* can vary among different subtribes and species of chlorophyllous tuberous terrestrial orchids (Chapter 2, 3). Similar patterns of fungal specificity *in vitro* were observed within genera (Chapter 3), though further investigation with a larger number of species could help determine a more conclusive answer. It should be noted that fungi utilised in these studies were identified using morphological (Chapter 2, 3) and DNA analysis (Chapter 4, 5, 6). Therefore, there is a clear potential for disparity between the different methods of fungal identification. With many orchidaceous fungi being considered part of the Fungi imperfecti, identification can prove to be very difficult using morphological methods, particularly within fungal genera (Rasmussen, 2006). Utilising advances in DNA analysis will provide far better accuracy in identification and when combined with analyses performed on morphologically identified strains can reveal surprising phylogenetic relationships, hitherto unknown (Weiss *et al*., 2004). The development of a standardised fungal identification method would certainly prove to be useful and provident; helping to eliminate any confusion between identification methods. In addition, older morphologically identified strains could also be identified and catalogued using DNA analysis, clarifying any uncertainties in their identification. Incorporating genetic analysis into future studies on the efficacy of fungi in the germination and growth of orchids would enable us to gain a better understanding of the specificity of orchid-fungal associations and how they change during the plant's lifetime.

The results of these experiments conform with those of previous studies investigating fungal specificity and compatibility in other terrestrial orchids (Warcup, 1981, 1988; Muir, 1989; Kulikov & Filippov, 2001; Bonnardeaux *et al.,* 2007). The relationship between orchids and their mycorrhiza still requires greater study and more work is needed to determine fungal associations and specificity of many orchid species. Further experiments on fungal compatibility can help validate current theories on orchid-fungal specificity, potentially revealing important relationships between orchids and fungal genera, and identifying viable fungal symbionts for orchid propagation. Such work would allow for a better overall understanding of orchid-mycorrhizal interactions and vital ecosystem processes. With this knowledge, it may be possible to achieve more success with propagating both chlorophyllous and achlorophyllous orchids,

especially those that demonstrate high fungal specificities. Greater study could also provide discoveries of many unknown interactions which take place under the soil's surface, perhaps highlighting new and novel ways of propagating and reintroducing rare orchid species.

The studies conducted in this thesis have demonstrated that optimal symbiotic propagation methods can be more effective in germination (Chapter 3, 4, 5, 6), subsequent protocorm development (Chapter 3, 4, 5) and *ex vitro* establishment of orchid plantlets (Chapter 4). These studies add a great deal more weight to the benefits of symbiotic propagation and concur with similar observations made by Muir, (1989); Rasmussen *et al*., (1990a); Anderson, (1991); Otero & Bayman, (2009) and Aggarwal & Zettler, (2010) with other orchid species. Symbiotic methods, were also shown to produce more advanced germinants in comparison to asymbiotic methods over the same time period (Chapter 3, 4, 5). Although asymbiotic methods, were generally not as successful in germination and development, this method still proved to be highly capable at inducing germination and producing orchid seedlings (Chapter 3, 4, 5, 6). With some orchid species, asymbiotic media proved to be the optimal method for producing viable plantlets (Chapter 3, 6). This work has shown that neither asymbiotic nor symbiotic propagation is obsolete and that optimal propagation methods in orchids can be highly genus or species specific (Chapter 3, 5, 6; Muir, 1989; Mckendrick, 2000; Rasmussen, 2006; Otero & Bayman, 2009; Dowling & Jusaitis, 2012). For an overview of the most successful propagation treatments for each species in this thesis, see Table 1.

The use of near mature seed can reduce the physical and chemical inhibitory factors affecting orchid seed embryos (Rasmussen, 2006). Utilising near mature seed capsules in future experiments may help improve germination percentages in the tested species and could be applied to species which prove difficult to germinate. It should be noted, however, that using immature, indehiscent seed capsules can result in lack of genetic diversity in germinants due to the potential culture of maternal capsule material which can produce clonal protocorms (Zettler *et al*., 2007).

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Table 1. Overall Orchid propagation success showing optimal media, fungal and seed treatments for each species. Maximum development stage and mean percentage germination is shown for each treatment at 5 weeks culture for *Microtis media;* 8 weeks for *Anacamptis morio*, Germany, *Dactylorhiza incarnata, Serapias spp.;* 12 weeks for *Anacamptis laxiflora*; *Ophrys apifera;* 20 weeks for *Anacamptis morio*, UK, *Dactylorhiza purpurella, Masdevallia spp., Stelis sp.* Peru and *Pleurothallopsis microptera*. * denotes mean number of protocorms per treatment. Total numbers of established mature plants *ex-vitro* is shown.

Species	Media	Fungal Strain	Seed Treatment	Treatment Time	Stage	% Germination	Mature *Ex-vitro*
Anacamptis laxiflora	H1	B1	VMS 70% EtOH, 1% NaOCl	1 min, 8 min	5	37.9	80
Anacamptis morio, Germany	H1	A36	SP 0.5% SDICN	20 minutes	5	8.8*	Insufficient Time
	H1	B1	SP 0.5% SDICN	20 minutes	5	8.9*	Insufficient Time
Anacamptis morio, UK	JI & H1	B1	VMS 70% EtOH, 1% NaOCl	1 min, 8 min	3	29.8	Insufficient Time
	H1	B1	VMS 70% EtOH, 1% NaOCl	1 min, 8 min	5	18.3	Insufficient Time
Dactylorhiza incarnata	H1	B1	SP 0.5% SDICN	20 minutes	3	15.5*	Insufficient Time
Dactylorhiza purpurella	H1 Cut	B1	VMS 70% EtOH, 1% NaOCl	1 min, 20 min	3	43.3	Insufficient Time
Masdevallia melanoxantha	P6668	-	VMS 70% EtOH, 1% NaOCl	1 min, 10 min	3	11.3	Insufficient Time
Masdevallia menatoi	1/2 P6668	-	VMS 70% EtOH, 1% NaOCl	1 min, 10 min	2	6.7	Insufficient Time
Masdevallia patchicutzae	1/2 KC	-	VMS 70% EtOH, 1% NaOCl	1 min, 10 min	2	0.8	Insufficient Time
Microtis media	H1	*Rhizoctonia solani*	SP 0.5% SDICN	20 minutes	*4*	16.75*	3
Ophrys apifera	H1	FOA8	VMS 70% EtOH, 1.5% NaOCl	1 min, 10 min	2	2.8	No
Pleurothallopsis microptera	1/2 P6668	-	VMS 70% EtOH, 1% NaOCl	1 min, 10 min	3	4.7	Insufficient Time
Serapias bergonii	M	-	SP 0.5% SDICN	20 minutes	2	11.9*	Insufficient Time
	H1	Q414	SP 0.5% SDICN	20 minutes	3	1.1*	Insufficient Time
Serapias parviflora	M	-	SP 0.5% SDICN	20 minutes	2	24.2*	Insufficient Time
	H1	Q414	SP 0.5% SDICN	20 minutes	3	2.9*	Insufficient Time
Stelis sp. Peru	1/2 P6668	-	VMS 70% EtOH, 1% NaOCl	1 min, 10 min	3	1.1	Insufficient Time

I have shown that substrate rugosity and complexity are important factors in the germination and development of tuberous terrestrial orchids in symbiotic culture *in vitro* and, with certain species, asymbiotic culture (Chapter 5). It was demonstrated that the presence of a fungal symbiont increased the magnitude of germination overall, but when there were cuts in the media this effect was also magnified. Whether these observations are an effect of substrate complexity on the fungus or a direct effect of substrate, or both, appears to be dependent on the species of orchid. To my knowledge there are no existing studies examining the effects of substrate complexity, rugosity and texture on the germination and development of orchids. This study gives some insight into the effects that influence germination in orchid seeds on natural soils and other surfaces, and provides a potential method to help optimise orchid propagation *in vitro*. Hopefully, this study will help prompt further research, to better determine the importance and function of substrate rugosity, complexity and texture on orchid-fungal interactions, their symbiosis and orchid propagation.

In Chapter 6, I have highlighted important factors in the propagation of Pleurothallid orchids and have demonstrated optimal asymbiotic media which can produce viable plantlets *in vitro*. These results expand on the limited body of research that exists on the propagation of these orchids, helping to inform future conservation efforts and horticultural production of this orchid subtribe. Further investigations focusing on optimisation of abiotic: seed scarification techniques and times; asymbiotic media constituents, and biotic variables: self-incompatibility of parent plants; seed longevity and the isolation of fungi from protocorms and seedlings *in situ*, could help to optimise propagation protocols for these orchids. Assessing, long term data from studies can also help ascertain effective treatments, allowing for the establishment of appropriate culture protocols.

Despite, the lack of germination observed in *N. nidus-avis* in Chapter 7, I have demonstrated the possibilities for the long term maintenance of microcosms with tree seedlings, fungi and soil communities. Although not a strict tripartite association, as described by Warcup (1985) and Yagame & Yamato (2012), fungal hyphae interacting with tree roots and orchid seed were observed, potentially forming a link between orchid seeds and tree roots. As no germination took place, these associations were not examined in greater detail. Considering

that these microcosms may contain a broad diversity of soil microorganisms and microfauna, it is likely that this environment would better simulate the environment that is experienced by orchid seed in a natural site. With further refinement and development, this method may prove to be a better representation of the soil communities and their interactions than a stricter experimental approach focusing on aseptic techniques for the introduction of different symbionts. Such complex communities may not necessarily assist with optimising the propagation of these orchids, as the complexity of potential abiotic and biotic factors affecting germination may be increased. However, it may also result in the discovery of factors which hitherto have yet to be investigated. Developing effective methods to investigate and conserve MH and HFDM orchids is an interesting topic and deserves greater research.

The results of Chapters: 3, 4 and 5 demonstrate the utility and advantage of symbiotic techniques for the propagation of orchid species. These methods have been used to successfully reintroduce a number of threatened orchids. Aggarwal & Zettler (2010) successfully reintroduced symbiotically grown seedlings of *Dactylorhiza hatagirea* (D.Don) Soó where previous attempts using asymbiotic seedlings had failed (Vij *et al*., 1995). Stewart *et al*., (2003) also utilised symbiotic techniques to successfully reintroduce the rare terrestrial orchid *Spiranthes brevilabris* Lindl. and Zettler *et al*., (2007) the epiphytic orchid *Epidendrum nocturnum* Jacquin into suitable natural habitats. Muir (1989); Anderson, (1991) and Wood & Ramsay (2004) also obtained similar success, introducing symbiotically grown plants into semi-natural settings. The use of symbiotic methods can also be used to introduce appropriate fungal communities into habitats with degraded soils, enabling seedling recruitment and hence, restoring their former function (Zettler, 1997a). With further research it may be possible to refine these techniques for the commercial production of orchids. This in tern may lead to improved propagation methods which can be applied to improving conservation outcomes.

Ultimately, this work has contributed to the fields of orchid-mycorrhizal studies, orchid propagation and conservation. Allowing for a greater understanding of orchid-fungi interactions and highlighting effective propagation protocols for a number of orchid species, potentially aiding future *ex situ* and *in situ* conservation efforts as well as horticultural production. Whilst these findings

can help to conserve orchids, it is of great importance that future conservation work aims to develop integrated, multi-disciplinary approaches, involving engagement of all potential parties and stakeholders, and combining strong educational arguments which can instil a lasting message – the vital importance of biodiversity. With this in mind, conservation efforts and resources should be primarily focused on improving the management of populations and habitats *in situ*, and actively mitigate against the negative impacts of human induced ecological changes. Ultimately, preventing the need for expensive, resource hungry *ex situ* conservation techniques.

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